

Research Paper

Comparative characterization of a temperature responsive gene (lactate dehydrogenase-B, *ldh-b*) in two congeneric tropical fish, *Lates calcarifer* and *Lates niloticus*

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Abstract

The characterization of candidate loci is a critical step in obtaining insight into adaptation and acclimation of organisms. In this study of two non-model tropical (to sub-tropical) congeneric perciformes (*Lates calcarifer* and *Lates niloticus*) we characterized both coding and non-coding regions of lactate dehydrogenase-B (*ldh-b*), a locus which exhibits temperature-adaptive differences among temperate and sub-tropical populations of the North American killifish *Fundulus heteroclitus*. *Ldh-b* was 5,004 and 3,527 bp in length in *L. calcarifer* and *L. niloticus*, respectively, with coding regions comprising 1,005 bp in both species. A high level of sequence homology existed between species for both coding and non-coding regions of *ldh-b* (> 97% homology), corresponding to a 98.5% amino acid sequence homology. All six known functional sites within the encoded protein sequence (LDH-B) were conserved between the two *Lates* species. Ten simple sequence repeat (SSR) motifs (mono-, di-, tri- and tetranucleotide) and thirty putative microRNA elements (miRNAs) were identified within introns 1, 2, 5 and 6 of both *Lates* species. Five single nucleotide polymorphisms (SNPs) were also identified within miRNA containing intron regions. Such SNPs are implicated in several complex human conditions and/or diseases (as demonstrated by extensive genome-wide association studies). This novel characterization serves as a platform to further examine how non-model species may respond to changes in their native temperatures, which are expected to increase by up to 6°C over the next century.

Key words: Barramundi; Nile perch; exons; introns; microRNAs; SSRs

Introduction

The lactate dehydrogenase-B enzyme (LDH-B) plays a critical role in maintaining aerobic metabolism by converting lactate, the major by-product of anaerobic glycolysis, to pyruvate via oxidation in the presence of its coenzyme nicotinamide adenine dinucleotide, (NADH) (reviewed by [1, 2]). LDH-B can also convert lactate directly to glucose via gluconeogenesis. This conversion of accumulating lactate from aerobic tissues (e.g. heart, skeletal muscle) occurs in

the liver and allows desired aerobic metabolic activity to be sustained for extended lengths of time (reviewed by [1, 2]). In addition to these metabolic functions, the LDH-B enzyme affects the oxygen binding affinity of hemoglobin (Hb) by altering intra-erythrocyte ATP concentrations of Hb in fish [3, 4]. This effect of LDH-B on Hb-O₂ binding affinity directly impacts delivery of Hb-bound oxygen to red muscle tissues and may therefore be an alternate mechanism by

which LDH-B affects sustainability of aerobic performance such as swimming performance in fish [2, 5, 6].

The dissolved oxygen which is available to aquatic organisms is inversely correlated with water temperature (Henry's Law) and thus leads to the potential for variability in the ability of Hb to uptake and transport oxygen under differing thermal regimes. In natural populations of aquatic organisms the genes involved in aerobic metabolism and oxygen transport, such as *ldh-b* and hemoglobin may therefore be subjected to strong selective pressure [3, 4, 7-11]. In fact, within thermally distinct populations of the temperate estuarine killifish *Fundulus heteroclitus* Hb-O₂ affinity varies together with intra-erythrocyte ATP concentrations dependent on which LDH-B isozyme (LDH-B^a or LDH-B^b) is fixed [3, 4]. Moreover, a significant difference in transcript abundance of *ldh-b* was observed in response to both thermal and aerobic stress in discrete North American *F. heteroclitus* populations, even after acclimation to a common temperature. This transcript response was linked to a one base pair mutation in the glucocorticoid responsive element (GRE) identified within the *ldh-b* 5' proximal promoter ([7, 10]; see also reviews by [1, 2]). In addition to the extensive characterization and investigation of *ldh-b* in *F. heteroclitus*, the translated protein of this candidate gene has also been characterized and investigated in other temperate fishes like rainbow trout, *Salmo gairdneri* [12, 13] and crested blenny, *Anoplarchus purpurascens* [14]. However, this gene has not been fully characterized in any tropical perciform to date and there have been no investigations into the role this gene may have in thermal acclimation or the capacity to cope with thermal stress in tropical fishes. Therefore, the *ldh-b* locus appears to be an ideal candidate gene for the investigation of thermal adaptation and/or acclimation to native thermal regimes [15] in non-model tropical fish..

As a first step in understanding the role *ldh-b* may have in thermal adaptation or acclimation of tropical fish species, we characterized this gene in two tropical congeners, the Australian barramundi (*Lates calcarifer*) and the African Nile perch (*Lates niloticus*). *Lates calcarifer* is a catadromous, protoandrous hermaphrodite, native to rivers, estuaries and shallow marine environments throughout northern Australia (25°S - 12°S) and the south-east Asian archipelago (13°N - 10°S) [16-18], while *Lates niloticus* originates from east African rivers and lakes (7°S - 27°N) [19]. These species were targeted in this study for four reasons. Firstly, they occupy a range of different thermal environments; secondly, they are commercially valuable; thirdly, they are well suited for ex-

perimental manipulation and, fourthly, they are congeners which allows for comparisons of nucleotide sequences between these congeneric tropical perciformes. In addition to the traditional characterization of coding regions (exons) we also characterize, for the first time in non-model fish, the non-coding regions (introns) of the *ldh-b* locus in these perciform species to establish if regulatory motifs and/or elements (simple sequence repeats (SSRs) or microRNAs (miRNAs)), which are known to be embedded in or encoded by non-coding regions, are present. Previous studies on a diverse range of taxa, including fish, have demonstrated the presence of such elements within introns of other genes, where they are implicated in regulation or silencing of transcription [20-28].

Materials and methods

Complementary DNA (cDNA) synthesis from hepatic messenger RNA (mRNA)

As no *ldh-b* sequence information was available for either species, total liver RNA was first required for reverse transcription to obtain a *Lates ldh-b* cDNA sequence for primer design. This cDNA sequence was necessary to enable the design of *ldh-b* specific primers targeting the non-coding (intron) sequences of the *ldh-b* locus that can only be obtained from genomic DNA. Liver RNA was targeted for this initial cDNA sequencing as this tissue exclusively expresses the LDH-B protein as opposed to the alternative isozymes, LDH-A or C, which are expressed in other, non-hepatic tissues [29-31]. Total RNA was extracted from snap-frozen livers from four Darwin Harbour, Northern Territory, *L. calcarifer* individuals using Trizol as per manufacturer's instructions (Invitrogen Australia Pty, Mount Waverley Victoria). Extracted hepatic RNA was treated with Turbo DNA-free (Ambion, Austin, TX, USA) to remove any remaining trace DNA contamination. To ensure that trace amounts of genomic DNA, if present, do not contribute to the synthesis of cDNA, intron spanning primers were designed (detailed below). RNA purity was verified by NanoDrop Spectrophotometer (Invitrogen Australia Pty, Mount Waverley Victoria) analyses of 260/230 and 260/280nm absorbance. Messenger RNA (mRNA) was reverse transcribed to generate cDNA immediately via IM-Prom II Reverse Transcriptase with Oligo dT₂₀ and random primers (Promega, Madison, WI USA), as per manufacturer's instructions.

Amplification of *ldh-b* from hepatic cDNA

Amplification and sequencing of *ldh-b* from the *L. calcarifer* hepatic cDNA was accomplished with general fish primers designed by aligning *ldh-b* coding

sequences from a taxonomically diverse range of fishes from the National Center for Biotechnology Information (NCBI) sequence database (GenBank) [32]. The following sequences were aligned and primers were designed based on conserved regions - Gadiformes *Trachyrincus murrayi* [AJ609235], *Merlangius merlangus* [AJ609234], *Gadus morhua* [AJ609233] *Coryphaenoides armatus* [AJ609232]; Cypriniformes *Danio rerio* and *Cyprinus carpio* [AY644476]; Cyprinodontiformes, *F. heteroclitus* [L43525], *F. heteroclitus* (D. Crawford personal communication); Squaliformes *Squalus acanthias* [AF059035]. Following the design of several primers the most specific *ldh-b* product was attained using the forward primer designed from

Gadiformes and Cypriniformes (ATGGCCTGTGCCGTCAGC) in conjunction with the reverse primer designed from Cypriniformes (TCTTTCAGGTCTTTCTGGAT), which anneal in exons 2 and 7 respectively. The remaining upstream sequence (exon 1 to 5' end of exon 2) was subsequently obtained using a previously published *ldh-b* forward primer for *F. heteroclitus* [33] and the reverse primers *L. calcarifer*-Intron2-R1 or *L. niloticus*-Intron2-R1 were designed from intron sequences subsequent to the initial cDNA amplification (Table 1). These intron sequences were obtained from genomic DNA amplification with *Lates*-specific *ldh-b* primers designed in exons 2 to 7.

Table 1. PCR and sequencing primers used to obtain *ldh-b* sequences in *Lates* species. Segments 1 to 5 refer to regions depicted in Figure 1. All primers anneal to gDNA (see Methods). Primers used for forward and reverse sequencing reactions only (nested primers) are indicated by (SeqF) and (SeqR), respectively. Final MgCl₂ concentrations in brackets refer to *L. niloticus* amplification requirements, which differed from that required for *L. calcarifer*.

Lates spp. <i>ldh-b</i> Primers	5' to 3' Sequence	MgCl ₂	T _a	Amplicon (bp)
Segment 1*				
<i>L. calcarifer</i> -Seg1-R1	GATTTAGACATGTCGTTCCCTCAG	1.5 - 2.5	61°C	2000
<i>L. calcarifer</i> -Seg1-R2	ATAATGACACCATCAATGTTTCACG	1.5 - 2.5	61°C	1000
<i>L. calcarifer</i> -Intron1 (SeqR)	ATGGATGAATGTCTCAATCAG	1.5 - 2.5	53°C	500
<i>L. calcarifer</i> -Intron2-R1	TCCGATAACAGAAGCACTCAC	1.5 - 2.5	55°C	1500
<i>L. niloticus</i> -Intron2-R1	TAATCACTCATGGCCTCGG	1.5 - 2.5	53°C	1300
<i>L. niloticus</i> -Intron1-R1	AACTGGAACTAATCTAGGCC	1.5 - 2.5	55°C	450
<i>L. niloticus</i> -Intron1 (SeqR)	TCAGGTTAGCACTGCTGC	1.5 - 2.5	51°C	350
Segment 2				
<i>L. calcarifer</i> -F1	TGATGGAGGATCGTCTGAAAGG	1.5 - 2.5	61°C	800
<i>L. calcarifer</i> -R1	TCCGCCATCAGGTAACGGAAG	1.5 - 2.5	61°C	800
Segment 3				
<i>L. calcarifer</i> -F2'	GTTGATGTGCTGACCTACGTC	1.5 - 2.5 [3.5 - 4.5]	59°C	1500
<i>L. calcarifer</i> -R2'	AGCCCTCAGCTTGATCACC	1.5 - 2.5 [3.5 - 4.5]	57°C	1500
Segment 4				
<i>L. calcarifer</i> -F3i	ACAGAGCTTCCACTGTATCAC	1.5 - 2.5	57°C	850
<i>L. calcarifer</i> -R3i	GCAAAAGGTTCTAGGCATGTA	1.5 - 2.5	59°C	850
<i>L. calcarifer</i> -F3	AGAAGCTGAACCCTGAGATCG	1.5 - 2.5 [3.5 - 4.5]	59°C	800
<i>L. calcarifer</i> -R3	TTCTGGATGCCCCACAGTGTG	1.5 - 2.5 [3.5 - 4.5]	61°C	800
Segment 5**				
<i>L. calcarifer</i> -F3i'	TGGTTGCTAGGATAAAGAATGTG	1.5 - 2.5	59°C	700
<i>L. calcarifer</i> -F3i (SeqF)	AGTTGTAAATAATTCAGGCATC	1.5 - 2.5	53°C	500
<i>L. niloticus</i> -Intron6-F1	ATGTGGATAGCCTAGCTTAGC	1.5 - 2.5	55°C	400

* and **: Published forward and reverse *F. heteroclitus* *ldh-b* primers [33] used in conjunction with designed primers to amplify terminal (5' and 3') segments in both *Lates* species, respectively.

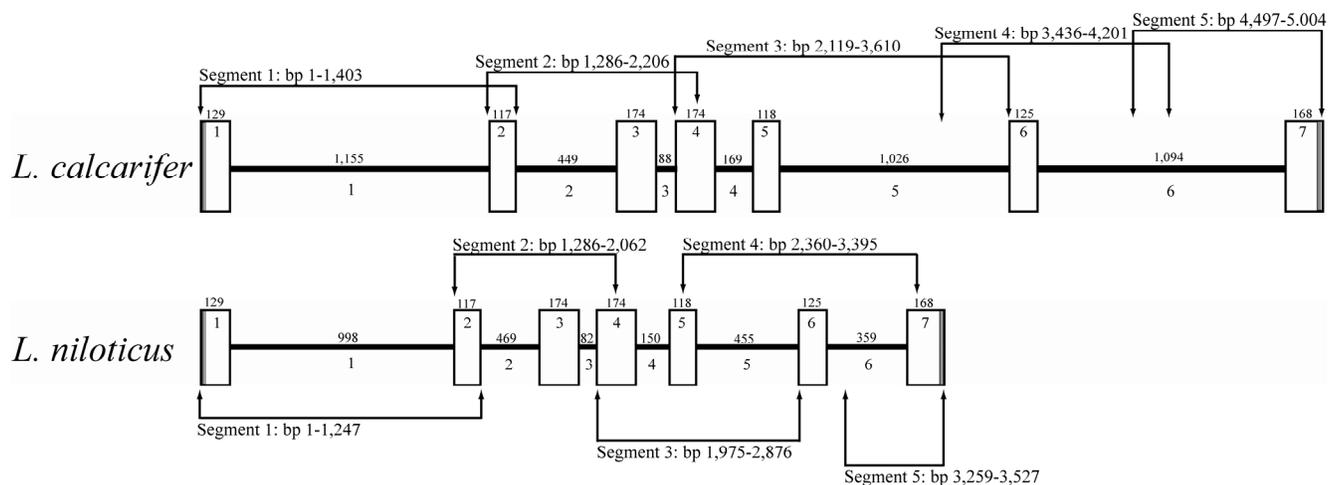


Fig. 1. Comparative *ldh-b* gene map of congeneric *Lates* species. Exon (white boxes) and intron (black bars) sizes in number of base pairs (bp) are given above their respective graphic representation. Sequential numbering within white boxes and below black bars is for exons and introns, respectively. Arrowhead lines indicate region amplified by specific primer pairs (as per Table 1) along with the size of each segment in number of base pairs.

All PCR reactions were conducted in the following manner: Amplification reactions (20 μ L) contained the following final concentrations: 1X Buffer [2.5 mM Tris pH 8.7, 5 mM KCl, 5 mM $(\text{NH}_4)_2\text{SO}_4$ containing 1.5 mM MgCl_2] (Qiagen, Doncaster, Victoria Australia) or 1x Buffer [2.5 mM Tris pH 8.7, 5 mM KCl, 5 mM $(\text{NH}_4)_2\text{SO}_4$ not containing 1.5 mM MgCl_2] (Bioline Pty Ltd., Alexandria, New South Wales Australia) (unless more was required as per Table 1), 250 μ M each dNTP, 250 nM each primer (Table 1), 10 ng gDNA template and 0.75 to 1.5 units of *Taq* Polymerase (Qiagen and Bioline Pty Ltd.). Thermal cycling was conducted on a MJR DNA Engine thermal cycler (Bio-Rad Laboratories Pty., Ltd., Gladesville, New South Wales) as follows: initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C denaturation for 30 sec, annealing at primer specific T_a for 30 sec (Table 1), 72°C extension for 30 to 90 sec depending on target fragment size with larger fragments (> 1,000 bp) requiring longer (> 60 sec) extension times (Table 1) and a final 72°C extension for 10 min. Melting temperature (T_m) was calculated via $(A/T \times 2 + G/C \times 4)$ method, with the annealing temperature (T_a) set at 5°C less than T_m , for all primer-pairs (Table 1).

Verification of *ldh-b* sequences amplified from hepatic cDNA

Ldh-b amplification from hepatic RNA derived cDNA with *Lates*-specific primers (see above) generated a strong single-band product of approximately 800 bp for all *L. calcarifer* examined ($n = 4$). Subsequent amplification of genomic DNA with the published forward (*F. heteroclitus*-F, [33]) and designed reverse

(*L. calcarifer*-Intron2-R1 and *L. niloticus*-Intron2-R1) primers resulted in a strong single-band product of approximately 1,500 bp and 1,300 bp (*L. calcarifer* and *L. niloticus*, respectively) containing the missing exon 1 and 5' end of exon 2 fragment. These products were precipitated with 120 μ L isopropanol (70%) for 15 minutes followed by a 500 μ L wash with 70% isopropanol prior to drying, re-suspension in water (10 μ L) and subsequent sequencing (Macrogen, Inc., South Korea). Sequences were edited in BioEdit [34] and a contig made to give the full length coding sequence (1,005 bp) and produce a consensus cDNA sequence. To check that the correct *ldh* gene homologue had been obtained the sequence was used in a BLAST search of GenBank and also directly aligned using ClustalW in Mega 3.1 [35] to those *ldh-b* sequences previously utilized for general fish primer design (see above). The obtained sequence shared 92% homology with *F. heteroclitus* LDH-B amino acid sequence, a level of homology that is well above that reported for *F. heteroclitus* LDH-C v. LDH-B and LDH-C v. LDH-A (78% and 70% homology, respectively) [30]). As a further check that the correct *ldh* gene homologue was obtained all nucleotide (and deduced amino acids) sequences were aligned with those of *Danio rerio* *ldh-b* [AF067202] and *ldh-a* [NM_131246] genes. These two gene homologues differ by 600 bp in length and 34.1% of the nucleotide sequences, respectively. The *L. calcarifer* *ldh-b* sequence obtained from hepatic cDNA most closely matched that of the *D. rerio* *ldh-b* gene homologue. Once the initial cDNA sequence was obtained from *L. calcarifer* the full characterization of the *ldh-b* locus from both *Lates* species (*L. calcarifer* and *L.*

niloticus) was accomplished via primer walking along genomic DNA from representative individuals of each species.

Study species and genomic DNA (gDNA) extraction

Genomic DNA was extracted from *L. calcarifer* samples collected from four locations within tropical Australia. Samples from Gladstone, Queensland (23°S, 151°E) and Darwin, Northern Territory (12°S, 130°E) were obtained directly from fish farms while Archer River (Cape York, Queensland: 13°S, 142°E) and Tully River (Tully, Queensland: 17°S, 145°E) samples originated from wild caught fish. *L. niloticus* was purchased as two imported frozen fillets at a local supermarket in Townsville, Queensland and therefore the exact geographical origin of the *L. niloticus* samples examined is unknown but assumed to be from one of the African Rift Valley lakes (4°N - 14°S) where a large export fishery of this species exists. *L. niloticus* fillets may or may not have been snap-frozen; regardless, extracted genomic DNA appeared to be of equal quality to the DNA from *L. calcarifer* samples. Fin-clips/muscle tissue were taken from all fish and preserved in ethanol (90%). DNA extractions were performed via proteinase-K digestion (20 mg/mL) in CTAB buffer at 60°C for 1 hr and DNA was subsequently cleaned with a salt and chloroform:isoamyl alcohol (24:1) procedure [36]. All extractions resulted in high molecular weight gDNA, as visualized on a 0.8% agarose gel, with quantities ranging from 20 - 100 ng/ μ L.

Amplification of the *ldh-b* locus from genomic DNA

Full length *ldh-b* gene sequences (including intron sequences) were obtained from genomic DNA extracts using the primers and primer specific PCR conditions outlined in Table 1 and Figure 1. In some cases different primers were required for amplification of *L. niloticus* and *L. calcarifer* introns (e.g. intron 5 & 6 in Figure 1). See Figure 1 for primer binding locations within the *ldh-b* locus. Thermocycling parameters were the same as that used for cDNA amplification with the exception of the annealing temperature and MgCl₂ concentration which varied as listed in Table 1. All PCR products obtained from both *L. calcarifer* and *L. niloticus* genomic DNA were verified as *ldh-b* in two ways: 1) searching the NCBI database (GenBank) via nucleotide (blastn) and protein (blastp) basic local alignment and search tool (b.l.a.s.t.) with resulting matches specific to LDH-B exclusively and 2) direct alignment with *F. heteroclitus* *ldh-b* nucleotide and LDH-B protein sequence. After confirmation of product identity full length gene sequences for each

individual (*L. calcarifer*: $n = 4$ and *L. niloticus*: $n = 2$) were assembled in BioEdit and the consensus nucleotide and deduced amino acid sequences from each species were aligned using ClustalW in Mega 3.1. The high level of conservation across individuals from both *Lates* species allowed for the compilation of *L. calcarifer* and *L. niloticus* consensus sequences. Consensus gene sequences for each species were submitted to GenBank under accession numbers [FJ439509] and [FJ439510] for *L. calcarifer* and *L. niloticus* respectively. Additionally, *L. calcarifer* *ldh-b* coding sequence isolated from hepatic mRNA was submitted to GenBank under accession number [FJ439507]. Deduced LDH-B amino acid sequences were manually assessed for the presence of variation within the NH₂-terminal arm (residues 1-20), coenzyme binding domain (residues 21-95 and 118-163), substrate binding domain (residues 164-333) and loop helix α D region (residues 96-117) [37].

Assessment of non-coding (intron) sequences for micro RNA (miRNA) and simple sequence repeat (SSR) motifs

Several recent studies have demonstrated intron sequences may contain simple sequence repeat (SSR) motifs of functional importance as they potentially bind regulatory machinery (e.g. promoters and/or enhancers) and may affect gene expression levels [25, 38]. Intron sequences from *ldh-b* of both *Lates* species were manually assessed for the presence of simple sequence repeat (SSR) motifs. In addition, short microRNA (miRNA) elements (21-23 bp) may be encoded by introns and these are believed to be spliced out of pre-messenger RNA (mRNA) subsequently targeting regions within the 3'UTR of actively expressed mRNAs regulating translation from mRNA transcripts to functional proteins [20-23, 39, 40]. Intron sequences from *Lates* spp. were therefore assessed for putative miRNA elements with the software package miRanda [40]. All presented miRNA elements are located within conserved intron regions of *Lates* spp. and matched known miRNA motifs from *Danio rerio* (zebrafish), *Takifugu rubripes* (Japanese pufferfish) and *Tetraodon nigroviridis* (spotted green pufferfish) (miRBase: [41-43]). Several of the identified elements also matched known *Xenopus tropicalis* (African frog) miRNA motifs (miRBase: [41-43]). A nucleotide match score (score), affinity to bind measure (energy [kcal mol⁻¹]), statistical assessment of match quality (z-score) and homology of motif to query intron sequence (percentage) were all calculated by miRanda for each matching miRNA element [40]. Threshold values were set to 100, -19 kcal mol⁻¹ and 5.0 for score, energy and z-score, respectively, to assess *ldh-b* for

miRNAs under strict parameters so as to avoid false-positive identifications ($\leq 5\%$) [40, 44, 45]. However, overly-stringent thresholds were avoided to maximize the likelihood of identifying putative miRNA as such elements have recently been documented in high abundance within eukaryotic genomes at an average of 100 binding sites per miRNA element, genome-wide [45].

Results and Discussion

Descriptive characterization of *ldh-b* coding nucleotide (exon) and deduced amino acid sequences

Variation in the primary sequence and level of gene expression of the *ldh-b* locus has been linked to differences in aerobic performance (reviewed by [1, 2]) and natural selection [7, 46] in the temperate (to sub-tropical) fish *F. heteroclitus*. The present study is the first to characterize the full length gene sequence of this important metabolic gene in two tropical perciform fish, namely *L. calcarifer* and *L. niloticus*. This locus consisted of 5,004 and 3,527 bp, of which 1,005 bp was coding, in these two species, respectively (Figure 1). Consistent with other fish species the *ldh-b* locus of both *L. calcarifer* and *L. niloticus* contained seven exons and six introns, with all seven *ldh-b* exons being conserved in size between the characterized *Lates* species (Figure 1). Additional coding nucleotide sequence comparison revealed 29 base differences between *L. calcarifer* and *L. niloticus* (2.9% divergence); however, the majority of these occurred in the third codon positions which lead to silent (i.e. synonymous) amino acid substitutions. This high level of conservation was expected between *Lates* species being that this locus is known to be under functional constraint due to its role in maintaining aerobic metabolism ([7, 46]; see also review by [2]). Moreover, the encoded enzyme (LDH-B) has also been shown to impact on hemoglobin-oxygen binding affinity in fish, with allozyme variants altering this critical interaction [3, 4].

Each residue of the post-translation modified protein sequences has a role in the specific functioning of the molecule, from its internal stability to its external functional interactions. Five amino acid differences (1.5%) are present between the LDH-B of both *Lates spp.* These variable amino-acids involve the substitution of threonine (T) for methionine (M), leucine (L) for alanine (A), valine (V) for isoleucine (I), lysine (K) for asparagine (N) and valine (V) for isoleucine (I) at residues 34, 35, 126, 127 and 147, respectively (Figure 2). All of these variable amino acid residues reside within the coenzyme (NADH) binding domain (residues 21-95 and 118-163), of LDH-B,

which is otherwise conserved between *Lates spp.* (Figure 2). This is unanticipated in such a conserved functional domain [30, 37]. Noteworthy are the two shifts occurring at residues 34 and 127 as these invoke changes in polarity (polar – non-polar) and acidity (neutral polar – basic polar) between *Lates* species, respectively (Figure 2). The effect of these amino acid shifts on the catalytic efficiency (k_{cat}) of LDH-B between *L. calcarifer* and *L. niloticus* is unknown and warrants further investigation. Conversely, the NH₂-terminal arm (residues 1-20), substrate binding domain (residues 164-333) and loop helix α D region (residues 96-117) [37], as well as the LDH-B substrate binding (residues 100, 107, 139, 170, 249) and proton acceptor (residue 194) sites found within [47], are conserved between both *Lates* species (Figure 2), as expected [30, 37].

Comparison between *Lates* species and *F. heteroclitus* full length LDH-B amino acid sequences revealed a relatively extensive divergence, with 30 amino acids (9.0% divergence) observed between these phylogenetically distant species (data not shown). The two residues demonstrated to have fixed differences between thermally and geographically distinct populations of *F. heteroclitus* were serine (S) *v.* alanine (A) and alanine (A) *v.* aspartic acid (D) at residues 185 and 311 for cold northern and warm southern populations, respectively (see review by [1, 2]). *L. calcarifer* and *L. niloticus* both possess serine (S) and aspartic acid (D) at residues 185 and 311, respectively (Figure 2). The deduced LDH-B amino acid sequence of both *Lates* species examined are therefore similar to the cold northern *F. heteroclitus* population at residue 185 and to the warm southern *F. heteroclitus* population at residue 311. These residues are located on the internal and external surfaces of the folded LDH-B protein, respectively ([48, 49]; see also reviews by [1, 2]). The former change (residue 185) has been hypothesized to be associated with a variation in thermal stability due to this residue being located at a hairpin turn in the center of the folded protein; whereas the latter change (residue 311) has been hypothesized to be associated with a variation in substrate binding affinity due to this residue being located on the external surface of the conformed protein ([48, 49]; see also reviews by [1, 2]). In light of these previous hypothetical explanations, future studies should strive to compare the structure and function (e.g. enzymatic activity, effect on Hb-O₂ binding affinity) of LDH-B in the *Lates* species in parallel to those studies conducted on within and among thermally discrete *F. heteroclitus* populations [4, 48, 49] and cod from two different temperatures [50].

ture investigations into the impact these indels may or may not have on *L. niloticus* *ldh-b* gene expression, as compared to the variation observed among thermally discrete *L. calcarifer* populations [53], is warranted.

Numerous simple sequence repeat (SSR) sequences were detected within *ldh-b* introns of both *Lates* species (Table 2). Intronic SSRs are of interest because they may regulate gene transcription, lead to abnormal splicing and disrupt export of mRNA to the cytoplasm [25, 38, 52, 54]. Four mononucleotide SSRs (T, C and A), ranging from 5 to 11 repeats, are present within intron 1 of both *Lates spp.* and these were conserved in size between species (Table 2). In addition to these, two mononucleotide repeats (T₁₀ and C₇₋₁₁) are also present exclusively in *L. calcarifer* introns 1 and 6, respectively (Table 2). One dinucleotide repeat (AC) is present at the same location within intron 6 of both *L. calcarifer* and *L. niloticus*, but it differs in repeat number between the species (AC₅ and AC₈, respectively) (Table 2). In addition there is one trinucleotide repeat (CAA) present within intron 1 of *L. niloticus* and *L. calcarifer* which varies between 3 and 4 repeats, respectively (Table 2). Another trinucleotide repeat (TCC₄) is present within a region in intron 2 and is conserved between both *Lates* species (Table 2). Noteworthy is that a similar (TCC₄) SSR was identified in the 5' flanking untranslated region (UTR) of the *ldh-b* locus in *F. heteroclitus* and, more importantly, that variation in repeat number of this SSR in the 5' UTR of *ldh-b* impacted the level of *ldh-b* transcription (i.e. gene expression) observed in thermally discrete *F. heteroclitus* populations [8, 10, 11, 29, 55]. Moreover, this region within the 5' proximal promoter of *F. heteroclitus*, in addition to the 6fp and Sp1 binding sites also identified, were concluded to be under functional constraint by way of a phylogenetic analysis on the nucleotide sequences of these 5' UTR regulatory motifs which clearly differentiated the cold northern from the warm southern population [7, 46]. Lastly, a tetranucleotide repeat (TGTA₄) is observed in a region of intron 6 exclusive to *L. calcarifer* (Table 2). The variation and potential functional role, if any, of these SSRs on *ldh-b* gene expression itself and/or on the greater transcriptome functionality within and among thermally discrete *L. calcarifer* and *L. niloticus* populations is currently unknown; however, further investigation into such potential implications on the transcriptome is warranted.

Numerous potential microRNA elements (miRNAs) were also identified within *Lates* species intron sequences (Table 3). The encoding of thirty putative miRNA elements was identified within conserved intron regions and these had a score, energy (kcal mol⁻¹), z-score and homology ranging from 102

to 140, -26.96 to -19.01, 5.12 to 14.94 and 61.76 to 87.5%, respectively (see Methods). Four of the putative miRNA elements identified (Table 3: *miR-let7b*, *miR-124*, *miR-181* and *miR-223*) have previously been associated with specific regulatory functions [21, 39, 56-59]. Of these, *miR-let7b* and *miR-223* knockout in mouse HeLa cells [59] and neutrophils [21] resulted in reduced expression of approx. 2,700 and approx. 3,800 proteins, respectively. Two additional sub-families (*d* and *e*) from the highly investigated *let-7* miRNA element family were identified within *Lates spp.* *ldh-b* introns (see Table 3), which provides additional data consistent with the widespread dispersal and high abundance of targets and/or functions of miRNA elements encoded by introns throughout the genome [21, 22, 45, 59]. Noteworthy is that the *dre-let-7* (*-b*, *-c*, *-d*) miRNA elements have also been recently identified within the 5' UTR of the *L. calcarifer* myostatin gene (*mstrn*) [60].

Five intronic single nucleotide polymorphisms (SNPs) also were identified in intronic regions of the *ldh-b* locus of individuals representing eight discrete *L. calcarifer* populations in a pilot population genetics screening [53]. Interestingly, these SNPs are present at sites where multiple miRNA elements overlap [53]. The fact that a single SNP can impact multiple putative miRNA elements concurrently may provide insight in regard to recent findings of genome-wide association studies, which show a relationship between such SNPs and variation in complex human behaviour (e.g. schizophrenia and bipolar disorder) and/or susceptibility to complex diseases (e.g. type 2 diabetes and Crohn's disease) [61-65].

Table 2. Simple sequence repeat (SSR) motifs present within non-coding intron sequences of *Lates* species.

SSR Motifs	Location	# of Repeats: <i>L. calcarifer</i>	# of Repeats: <i>L. niloticus</i>
Mononucleotide			
T [^]	Intron 1	10	-
C	Intron 1	6	5
C	Intron 1	8	5
T	Intron 5	8	5
A	Intron 6	7	8
C [^]	Intron 6	7 - 11	-
Dinucleotide			
AC	Intron 6	5	8
Trinucleotide			
CAA [*]	Intron 1	4	3
TCC [*]	Intron 2	4	4
Tetranucleotide			
TGTA [^]	Intron 6	4	-

[^]: Indicates repeats exclusive to *L. calcarifer*. ^{*}: Indicates a simple sequence repeat motif identified within the 5' proximal promoter of *ldh-b* in *F. heteroclitus*, of which the repeat number impacts *ldh-b* expression [7, 11].

Table 3. Putative microRNA (miRNA) motifs identified within *ldh-b* intron sequences of *Lates* spp. Score: nucleotide match score; Energy: affinity to bind measure; Z-Score: statistical assessment of match quality; Homology: motif homology to query intron sequence.

miRNA Family	Sub-families	Score	Energy (kcal mol ⁻¹)	Z-Score	Homology (%)
let-7	b, d, e	104 to 128	-22.65 to -19.17	5.12 to 11.04	65.52 to 86.36
miR-10	b, c, d	102 to 127	-24.50 to -19.01	5.75 to 10.02	65.38 to 76.00
miR-15	a	109 to 128	-21.88 to -20.64	7.29 to 12.20	66.67 to 82.61
miR-23	a, b	113 to 128	-20.19 to -19.53	10.02 to 12.92	75.00 to 80.00
miR-24	-	105 to 112	-19.81 to -19.29	6.72 to 8.75	75.00 to 80.00
miR-25	-	113 to 122	-25.79 to -20.77	8.52 to 10.96	72.41 to 85.71
miR-27	c, e	106 to 130	-24.44 to -19.36	6.91 to 13.77	76.92 to 83.33
miR-101	a	109 to 110	-20.02 to -19.23	7.18 to 7.91	68.97 to 73.91
miR-103	-	111 to 114	-22.68 to -19.15	6.84 to 8.19	73.08 to 87.50
miR-107	-	104 to 110	-21.16 to -20.59	5.56 to 7.31	67.86 to 70.37
miR-122	-	109 to 130	-24.44 to -19.67	6.35 to 12.72	64.71 to 87.50
miR-124	-	126 to 133	-21.18 to -19.68	11.54 to 13.73	71.43 to 72.41
miR-125	a, b	102 to 118	-21.84 to -19.47	6.10 to 10.16	64.00 to 76.92
miR-138	-	106 to 117	-20.40 to -19.19	7.11 to 9.45	65.62 to 81.82
miR-140	-	104 to 115	-23.55 to -19.32	6.17 to 9.55	70.00 to 79.17
miR-148	-	109 to 115	-23.74 to -19.82	7.21 to 9.3	86.36
miR-152	-	102 to 140	-25.95 to -19.30	5.75 to 14.94	69.57 to 84.00
miR-181	a, b	103 to 112	-21.00 to -19.61	5.25 to 8.89	61.76 to 76.00
miR-183	-	118 to 127	-26.75 to -19.73	8.69 to 11.3	68.75 to 77.78
miR-184	-	101 to 128	-24.99 to -20.76	5.73 to 12.28	70.37 to 80.00
miR-187	-	103 to 122	-22.26 to -19.02	6.93 to 13.45	73.08 to 81.82
miR-196	a, b	107 to 127	-22.22 to -19.98	6.51 to 11.52	73.08 to 83.33
miR-202	-	107 to 126	-24.76 to -19.41	7.42 to 11.74	68.97 to 82.61
miR-210	-	102 to 127	-26.96 to -19.62	6.08 to 12.54	68.00 to 82.61
miR-214	-	102 to 114	-21.45 to -19.68	6.78 to 9.93	70.37 to 77.27
miR-216	b	111 to 112	-19.16 to -19.15	7.98 to 8.68	72.41 to 77.78
miR-217	-	112 to 132	-26.17 to -19.47	7.67 to 12.26	70.37 to 84.00
miR-221	-	105 to 133	-21.67 to -19.42	5.59 to 12.58	70.00 to 81.48
miR-222	-	105 to 127	-25.88 to -19.36	5.13 to 10.61	70.00 to 76.92
miR-338	-	121	-19.65	10.32 to 11.04	75.00

Evidence for selective constraint on non-coding (intron) sequences

The putative encoding of known functional motifs and/or elements (SSRs and miRNAs) by intronic regions of loci, in addition to the even distribution of such motifs and/or elements throughout longer introns [66], provides evidence that functional constraint is arguably acting on these historically less characterized non-coding regions. Indeed, such functional constraint is more likely to occur for introns of loci whose coding nucleotide or amino acid sequences are known to be under selection [22, 51, 52]. More specifically, longer introns (> 87 bp) have been found to exhibit less divergence than shorter introns (< 87 bp) because of either an increased likelihood of embedded functional motifs being present in longer introns or the potential impact any mutations may have on the secondary structure of precursor messenger RNA (pre-mRNA) [66]. A recent pairwise and

cross-taxa comparison of intron sequences between three mammalian species (human, whale and seal) revealed sequence homologies 14% and 12% higher, respectively, than those expected from a neutral model of evolution based on expected rates of substitution for non-coding DNA, further suggesting functional constraint acting upon non-coding intronic sequences [51]. Moreover, the existence of numerous intron motifs and/or elements is known to be essential for the functioning of complex multi-cellular organisms, as they permit a two-fold regulatory system in eukaryotic organisms: one for the transcriptome [20, 22, 25, 26, 28, 67] and one for the proteome [21, 39, 59]. Regardless, further research is required to determine if these motifs and/or elements (SSRs and/or miRNAs) have similar target sites and/or impacts on the transcriptome (i.e. gene expression) and/or the proteome (i.e. gene silencing) within and among *Lates* species and/or fish in general.

Conclusions

The *ldh-b* locus was found to be highly conserved between two tropical perciformes, *L. calcarifer* and *L. niloticus*, with just 2.9% divergence of coding regions and five amino acid differences between deduced LDH-B protein sequences. Variation within the co-enzyme binding domains (residues 21-95 and 118-163) of the deduced protein sequences may possibly confer a variation in the specific catalytic efficiency of LDH-B in geographically isolated *L. calcarifer* and *L. niloticus* and warrants further exploration. Non-coding (intron) sequences of the *ldh-b* locus in both *Lates* species were as conserved as the coding regions of this gene, despite comprising 72.5 to 80% of the entire gene sequence. Ten SSR motifs and thirty putative miRNA elements were observed within the introns of the *ldh-b* locus in both *Lates* species. These putative regulatory elements and/or motifs warrant further investigation for their potentially functional importance in the regulation and/or expression of *ldh-b* or other constituent loci contributing to the transcriptome and proteome. The characterization of the *ldh-b* locus has spawned additional studies, one of which confirmed that *ldh-b* expression differs significantly between *L. calcarifer* populations from different temperature environments, suggesting that *ldh-b* is adaptive in this species [53], as was shown for the temperate (to sub-tropical) killifish *Fundulus heteroclitus* [7, 46]. These findings, in conjunction with a pilot population genetics screening of the *ldh-b* locus among and within discrete *L. calcarifer* populations [53], are suggestive of thermal adaptation occurring within this tropical estuarine species. Moreover, future studies on other non-model tropical species are also permitted by the *ldh-b* characterization presented herein. Indeed, future studies of this adaptive locus in other non-model tropical species will provide insight into the response of thermally sensitive species (living at the edge of their thermal maxima) to changes in their native temperatures, which are expected to increase by up to 6°C over the next century.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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