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Differential expression of myogenic regulatory factor MyoD in pacu skeletal muscle (*Piaractus mesopotamicus* Holmberg 1887: Serrasalminae, Characidae, Teleostei) during juvenile and adult growth phases

Fernanda Losi Alves de Almeida [a,b], Robson Francisco Carvalho [a], Danillo Pinhal [a], Carlos Roberto Padovani [c], Cesar Martins [a], Maeli Dal Pai-Silva [a,*]

[a] *UNESP, Institute of Biosciences, Department of Morphology, 18618-000 Botucatu, São Paulo, Brazil*
[b] *UNICAMP, Department of Cellular Biology, Institute of Biology, 13084-971 Campinas, São Paulo, Brazil*
[c] *UNESP, Department of Biostatistics, 18618-000 Botucatu, SP, Brazil*

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ABSTRACT

Skeletal muscle is the edible part of the fish. It grows by hypertrophy and hyperplasia, events regulated by differential expression of myogenic regulatory factors (MRFs). The study of muscle growth mechanisms in fish is very important in fish farming development. Pacu (*Piaractus mesopotamicus*) is one of the most important food species farmed in Brazil and has been extensively used in Brazilian aquaculture programs. The aim of this study was to analyze hyperplasia and hypertrophy and the MRF MyoD expression pattern in skeletal muscle of pacu (*P. mesopotamicus*) during juvenile and adult growth stages. Juvenile ($n = 5$) and adult ($n = 5$) fish were anaesthetized, sacrificed, and weight (g) and total length (cm) determined. White dorsal region muscle samples were collected and immersed in liquid nitrogen. Transverse sections (10 μm thick) were stained with Haematoxilin–Eosin (HE) for morphological and morphometric analysis. Smallest fiber diameter from 100 muscle fibers per animal was calculated in each growth phase. These fibers were grouped into three classes (<20, 20–50, and >50 μm) to evaluate hypertrophy and hyperplasia in white skeletal muscle. MyoD gene expression was determined by semi-quantitative RT-PCR. PCR products were cloned and sequenced. Juvenile and adult pacu skeletal muscle had similar morphology. The large number of <20 μm diameter muscle fibers observed in juvenile fish confirms active hyperplasia. In adult fish, most fibers were over 50 μm diameter and denote more intense muscle fiber hypertrophy. The MyoD mRNA level in juveniles was higher than in adults. A consensus partial sequence for MyoD gene (338 base pairs) was obtained. The Pacu MyoD nucleotide sequence displayed high similarity several vertebrates, including teleosts. The differential MyoD gene expression observed in pacu white muscle is possibly related to differences in growth patterns during the phases analyzed, with hyperplasia predominant in juveniles and hypertrophy in adult fish. These results should provide a foundation for understanding the molecular control of skeletal muscle growth in economically important Brazilian species, with a view to improving production quality.

1. Introduction

Fish skeletal muscle is predominantly composed of white muscle, which never comprises less than 70% of the bulk of myotomal muscle and constitutes the edible part of the fish (Zhang et al., 1996). White muscle is made up of glycolytic metabolism and fast contracting muscle fibers (Driedzic and Hochachka, 1976) used in fast swimming such as predation and escape behavior (Altringham and Johnston, 1988). Red muscle forms a thin superficial layer, generally making up less than 30% of total musculature (Greer-Walker and Pull, 1975; Hoyle et al., 1986; Luther et al., 1995). Red muscle fibers display aerobic metabolism and slow contraction; they are associated with slow cruise swimming such as migration and foraging (Bone, 1966; Jonhston et al., 1977). There is an intermediate layer between red and white musculature which has intermediate characteristics (Sänger and Stoiber, 2001).

Fish muscle growth is a plastic mechanism involving populations of myogenic precursor cells, also called adult myoblast or myosatellite cells (Johnston, 1999). These cells provide the

* Corresponding author. Tel.: +55 14 3811 6264; fax: +55 14 3811 6264.
E-mail address: dpsilva@ibb.unesp.br (M. Dal Pai-Silva).

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essential nuclei for new muscle fiber formation (hyperplasia) and hypertrophy (Koumans and Akster, 1995). During hypertrophic growth, as fibers expand they absorb myoblast nuclei in order to maintain a relatively constant nuclear to cytoplasmatic ratio (Koumans et al., 1994). In hyperplasic growth, new fibers form on the surface of existing fibers by myoblasts fusing to form multinucleated myotubes (Johnston, 1999; Rowlerson and Veggetti, 2001). Final body weight depends on both hypertrophy and hyperplasia in muscle growth. In large, fast growing fish, hyperplasia is particularly active during the larval and juvenile stages (Weatherley and Gill, 1984). In small, slow-growing species, its contribution during adult life is low and muscle growth primarily involves hypertrophy of fibers formed in the embryo and during the early larval stage (Weatherley and Gill, 1984; Weatherley et al., 1988).

Hyperplasia and hypertrophy mechanisms are regulated by the sequential expression of members of the myogenic regulatory factor (MRF) family which include MyoD, Myf5, Myogenin, and MRF4 (Watabe, 1999, 2001). MRFs are transcription factors that share a highly conserved central region termed the basic helix–loop–helix (bHLH) domain (Edmonson and Olson, 1993) which mediates sequence-specific DNA binding called E-box, which is found in the promoters regions of many skeletal muscle specific genes (Lassar et al., 1989; Murre et al., 1989; Blackwell and Weintraub, 1990).

The primary MRFs, MyoD and Myf5, direct proliferating myogenic progenitor cells towards a myogenic lineage, whereas the secondary MRFs, Myogenin and MRF4, control the differentiation and fusion of myoblasts to form myofibers (Megeney and Rudnicki, 1995; Rudnicki and Jaenisch, 1995; Watabe, 1999). As per Johansen and Overturf (2005), during the initial growth phases, myoblast proliferation and hyperplasia can be inferred by the high expression of MyoD and Myf5, whereas Myogenin and MRF4 expression can be related to myoblast differentiation and hypertrophy, more intense during adult growth phase. Understanding the molecular control of postembryonic muscle growth in fish is one of the most important factors in successful aquaculture which accounts for 30% of global fish production (Tan et al., 2006).

The neotropical characid pacu (*Piaractus mesopotamicus*) has been extensively used in Brazilian aquaculture programs (Hernandez, 1989; Urbinati and Gonçalves, 2005). It is an omnivorous fish and is one of the most important food species farmed in the Pantanal wetlands area of the Paraná-Paraguai basin (Godoy, 1975). It is an autochthon species with immense economic importance in South American commercial fishing (Goulding, 1981). Pacu is a fast growing fish with a large final size (Bernardino and Colares de Melo, 1989) which depends on hyperplastic and hypertrophic muscle growth mechanisms (Dal Pai et al., 2000).

Since there are no studies focusing on the molecular basis of muscle growth regulation in pacu, the aim of our study was to investigate hyperplasia and hypertrophy and the MRF MyoD mRNA expression pattern in pacu skeletal muscle during juvenile and adult growth phases.

2. Materials and methods

2.1. Fish samples

Specimens of pacu (*P. mesopotamicus*) were obtained from the Aquaculture Center, UNESP, in Jaboticabal, São Paulo State, Brazil. Two development stages, juvenile ($n = 5$) and adult ($n = 5$), were used in this study. Fish were anaesthetized with MS-222 (Tricaine Methanensulfonate; Sigma–Aldrich Corporation, St. Louis, MO, USA) and sacrificed. Body weight (g) and total length (cm) were determined.

2.2. Morphologic and morphometric analysis

In each development stage, white muscle samples from the dorsal region ($n = 5$) were collected, immersed in *n*-hexane, cooled in liquid nitrogen (−159 °C), and then stored at −80 °C in a freezer until sectioning. Transverse 10 μm thick sections were obtained in a −20 °C cryostat and stained with Haematoxilin–Eosin (HE) (Bancroft and Steven, 1990). This was used to evaluate muscle morphology and calculate fiber diameter (Dubowitz and Brooke, 1973).

Fiber cross-section diameter (μm) was estimated by measuring 100 white muscle fibers from each animal per group using a compound microscope attached to a computerized imaging analysis system (Leica Qwin, Wetzlar, Germany) using the smallest diameter method (Dubowitz and Brooke, 1973). The smallest fiber diameter was used to avoid any errors that might have been caused by cross-sections not being completely true (Dubowitz and Brooke, 1973). White muscle fibers were grouped into three diameter classes: <20, 20–50 and >50 μm, based on Valente et al. (1999). Muscle fiber frequency was expressed as the number of fibers from each diameter class relative to the total number of fibers measured.

2.3. Semi-quantitative RT-PCR analysis of mRNA for MyoD gene

Total RNA was extracted from frozen juvenile and adult white muscle samples from each animal with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), based on the guanidine thiocyanate method (Chomczynski and Sacchi, 1987). Frozen muscle samples were mechanically homogenized on ice in 1 mL of ice-cold TRIzol reagent. Total RNA was solubilized in RNase-free water and quantified by measuring optical density (OD) at 260 nm. RNA purity was ensured by obtaining a 260/280 nm OD ratio >1.70. These total RNA samples were then PCR amplified to ensure no DNA contamination of RNA. Four micrograms of RNA were reverse transcribed with random hexamer primers and First Strand cDNA Synthesis Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) in a total volume of 33 μL, according to standard methods. One microliter of cDNA was then amplified using 0.2 mM of each primer (Table 1), 1× PCR buffer minus Mg, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphates, and one unit of Platinum Taq DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) in a final volume of 25 μL.

Primer pairs for MyoD were designed with reference to cDNA nucleotide sequence from *Ictalurus furcatus* (GenBank accession no. AY562555) (Table 1). PCR amplifications for MyoD gene were

Table 1
Nucleotide sequences and annealing temperature (T_A) of primers used for Polymerase Chain Reaction RT-PCR amplification of MyoD and 18S rRNA genes

Genes	Sequence (5′ → 3′)	T_A (°C)	Cycles	Size of amplified fragment (bp)
MyoD	Forward: CTAACCAGAGGCTGCCHAAG Reverse: CACGATGCTGGACAGACAGT	55	35	288
18S rRNA	Forward: TACCACATCCAAAGAAGGCAG Reverse: TCGATCCCGAGATCCAACTAC	57	32	245

T_A: Annealing temperature; bp: base pairs.

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carried out for 3 min at 94 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, 1.5 min of annealing at 55 °C, 2 min extension at 72 °C, and an additional 5 min extension step. A set of primers designed from the 18S ribosomal RNA (rRNA) consensus fish sequences were used to amplify a segment of the 18S rRNA gene (Tom et al., 2004) (Table 1). This gene was used as the housekeeping gene in semi-quantitative RT-PCR analysis. PCR amplifications for 18S rRNA gene were carried out for 2 min at 94 °C, followed by 32 cycles of denaturation for 1 min at 94 °C, 1 min of annealing at 57 °C, 1 min of extension at 72 °C and an additional 5 min extension step.

Preliminary experiments were conducted to determine the appropriate number of PCR cycles so that amplification product was clearly visible on an agarose gel and could be quantified, but also to assure that amplification was in the exponential range and had not reached a plateau. The number of cycles tested was 28, 30, 32, 34, 35 and 36 for both genes studied.

PCR products were verified by cloning and sequencing; cDNA from each muscle for both juvenile and adult groups were amplified simultaneously using aliquots from the same PCR mixture. After PCR amplification, 10 μL of each reaction underwent electrophoresis on 1% agarose gels and was stained with Sybr Safe (Invitrogen Life Technologies, São Paulo, SP, Brazil). The bands were visualized under UV illumination (Hoefer UV-25) and the gel image was retrieved using the EDAS program (Electrophoresis Documentation and Analysis System 120-Kodak Digital Science 1D). Bands corresponding to each gene were quantified in arbitrary units as optical density × band area, using Kodak one-dimensional (1-D) image analysis system (Eastman Kodak, Rochester, NY). PCR signals were normalized to the 18S rRNA signal of the corresponding RT product to provide a semi-quantitative estimate of MyoD gene expression. The PCR products were run in duplicate on different gels for each gene and results averaged.

2.4. cDNA cloning of MyoD

All amplified MyoD cDNA fragments were inserted into PGEM-T plasmids (Promega Corporation, Madison, WI, USA) which were used to transform competent *Escherichia coli* strain DH5α cells (Invitrogen Life Technologies, Carlsbad, CA, USA). The positive clones were sequenced on an ABI Prism 377 automatic DNA sequencer (PerkinElmer) with a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare Bio-Sciences) as per manufacturer instructions.

2.5. Nucleotide sequence analysis

Nucleotide sequences obtained from cloned MyoD-cDNA were subjected to BLASTN (Altschul et al., 1997) searches at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/blast) to confirm putative similarity with MyoD gene. MyoD consensus sequence was obtained using the Bioedit computer program (Hall, 1999). In addition, MyoD sequences from different vertebrates obtained from NCBI, were aligned using ClustalW software (http://www.ebi.ac.uk/clustalw/) (Thompson et al., 1994) and submitted to Neighbor-Joining (NJ) analyses employing the Kimura-2-parameter genetic distance model (Kimura, 1980) using MEGA 3.1 software (Kumar et al., 2004). Bootstrap resampling (Felsenstein, 1985) was applied to assess support for individual nodes using 1000 replicates.

2.6. Statistical analysis

Body weight data were expressed as median ± total semi-amplitude. The non-parametric Mann–Whitney test was used for weight analysis (Norman and Streiner, 1993). Total body length data were expressed as mean ± S.D. and analysis was performed using the Student's unpaired *t*-test (Norman and Streiner, 1993).

White muscle fiber diameters and semi-quantitative RT-PCR data were expressed as mean ± S.D. White muscle fiber diameters were analyzed using the Goodman test (Goodman, 1964, 1965). In semi-quantitative RT-PCR analysis, comparisons between groups were performed using the Student's unpaired *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Anatomical data

Median and total semi-amplitude weight was 16.45 ± 9.37 g for juvenile and 768.00 ± 238.50 g for adult fish ($p < 0.001$). Mean and S.D. of total length was 10.29 ± 1.29 cm for juvenile and 35.36 ± 2.8 cm for adult fish ($p < 0.001$).

3.2. Morphologic and morphometric analysis

HE stain showed white skeletal muscle making up most of the muscle mass in both juvenile and adult fish. This muscle consisted of round or polygonal muscle fibers separated by fine septa of connective tissue, the endomysium. Thicker septa of connective tissue separated muscle fibers into fascicles and making up the perimysium. Muscle fibers were distributed in a mosaic pattern characterizing fibers of different diameters (Fig. 1).

Frequency distribution of <20 μm diameter white muscle fibers in juvenile fish was significantly higher than in adults. Frequency

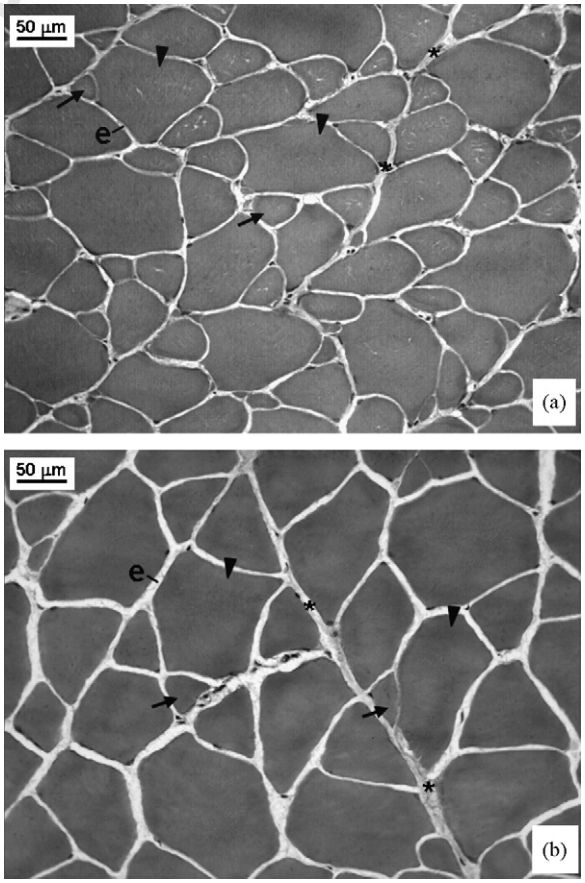

Fig. 1. Transverse sections of white skeletal muscle of juvenile (a) and adult (b) pacu (***Piaractus mesopotamicus***). A mosaic pattern of different muscle fibers diameters composed of small fibers (arrows) between large fibers (arrowhead) can be observed. Perimysium (*). Endomysium (e). HE. Scale bars: 50 μm.

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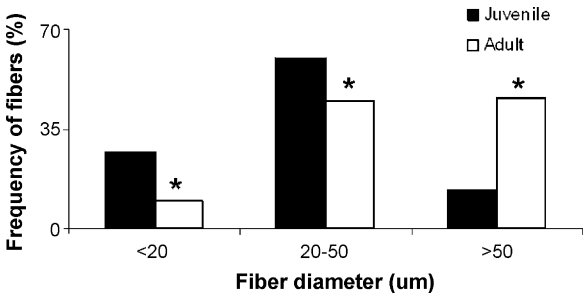

Fig. 2. White muscle fiber diameter distribution in juvenile and adult pacu (***Piaractus mesopotamicus***). Columns represent white fiber frequencies (%) in each group (asterisks in the column show size classes with significant variation; $p < 0.05$).

distribution of >20 to <50 μm diameter white muscle fibers and >50 μm diameter fibers were significantly higher in adults than juveniles (Fig. 2).

3.3. MyoD mRNA levels estimated by semi-quantitative RT-PCR

PCR amplification of pacu cDNA for MyoD gene generated one band of approximately 300 base pairs (bp), and for pacu cDNA with the 18S rRNA gene primer set generated one band of approximately 250 bp (Fig. 3a). Estimated MyoD mRNA level decreased in the adult group when compared to juveniles (juvenile 0.50 ± 0.04 vs. adult 0.26 ± 0.05; $p < 0.05$) (Fig. 3b).

3.4. MyoD nucleotide sequence analysis

The PCR products obtained with the MyoD set of primers were cloned, and a total of six clones (three from juvenile and three from adult muscle samples) were sequenced. A consensus sequence was produced from these clones and the exact total length of the cDNA fragment was 338 bp for MyoD (Fig. 4). The MyoD-cDNA consensus nucleotide sequence was subject to Blastn and showed high similarity to MyoD of several vertebrates, including teleosts *Danio rerio* (Perciformes), *Ictalurus punctatus*, and *Ameiurus catus* (Siluriformes). Phylogenetic analysis clustered the fish MyoD sequences into 100% of the recovered trees (Fig. 5).

4. Discussion

This study is the first description of differential myogenic regulatory factor MyoD expression in skeletal muscle of *P. mesopotamicus* during the juvenile and adult growth phases. MyoD mRNA level was significantly higher in juvenile than in adult fish.

Morphological examination of skeletal muscle in pacu (*P. mesopotamicus*) showed the majority of musculature in both phases composed of deep white compartment. This musculature contains muscle mass with considerable economic significance (Zhang et al., 1996). White muscle morphology in both stages was similar to other fish species (Fernandez et al., 2000; Dal Pai-Silva et al., 2003a,b; Aguiar et al., 2005). Although compartmentalized

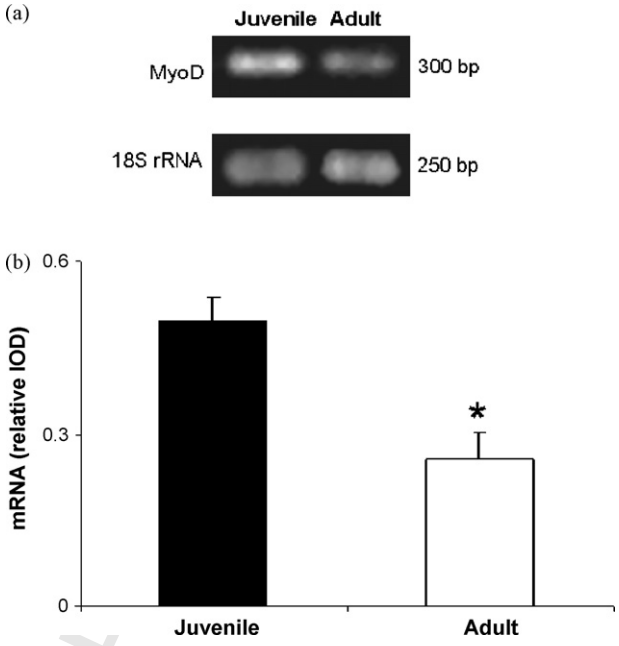

Fig. 3. MyoD and 18S rRNA representative RT-PCR profiles (a) and RNA content estimated by RT-PCR (b) from white muscles in juvenile and adult fish. MyoD gene expression was normalized to the 18S rRNA gene signal from the same RT product. Normalized data are expressed as means ± SE. $^{*}p < 0.05$ statistical significance.

muscle fiber distribution is common in fish (Scapolo et al., 1988; Veggetti et al., 1993; Galloway et al., 1999; Johnston, 1999), fiber distribution pattern can vary according to species (Te Kronnie et al., 1983; Dal Pai-Silva et al., 1995a,b) and growth stage (Dal Pai-Silva et al., 2003a,b).

As previously described by Dal Pai et al. (2000), juvenile and adult phase pacu muscle fibers have a mosaic pattern distribution, characterized by different diameter fibers; this has also been seen in others fish species (Rowlerson and Veggetti, 2001). Frequency distribution of <20 μm diameter muscle fibers was significantly higher in juvenile fish and the frequency of >50 μm diameter fibers was significantly higher in adult fish.

The large number of <20 μm diameter muscle fibers observed in juvenile fish confirm an active hyperplastic growth process in skeletal muscle during this developmental stage (Valente et al., 1999; Rowlerson and Veggetti, 2001). Hyperplastic growth in teleosts is mainly in two waves (Rowlerson and Veggetti, 2001). The first is a continuation of embryonic myogenesis and takes place during part of larval life generating new fibers along a germinal or proliferative zone (Usher et al., 1994); it is responsible for thickening muscle mass in early development stages (Rowlerson and Veggetti, 2001; Johnston et al., 2003). This event is known as stratified hyperplasia and occurs in most of fish species (Johnston, 1999). The second, mosaic hyperplasia, occurs in fish which grow to large sizes, such as the pacu, and new fiber

CTAACCAGAGGCTGCCHAAGGTGGAGATCCTGAGGARCGCCATCAGCTACATCGAGTCCCTG
CAGGCTCTGCTGCGCAGCCAGGAGGACTCCTACTACCCCGTCCTGGATCAGTACAGCGGGGA
CTCGGACGCGTCCAGCCCCAGATCCAACTGCTCCGATGGCATGGTGAGGACACTGAAGCGGC
TCTGACGCGTCCTTGATTGACCAGTAGATAGATAGTGTAGTGGGTGAATCCGTCGCCTGGCT
GTTTTTATTGTGTTTTGATTTCCTCCAGGTGACGTGCGGAGCAGCAAGTCCACAGTGGTGTC
CAGTCTGGACTGTCTGTCCAGCATCGTG

Q3 Fig. 4. Consensus sequence (5′ → 3′) for MyoD obtained from alignment of cloned cDNA sequences. Primer annealing regions are shadowed. H: Adenine, thiamine, or cytosine. R: Adenine or guanine.

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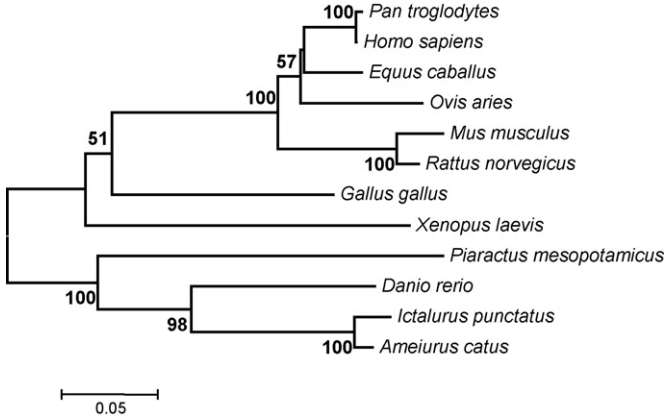

Fig. 5. Recovered Neighbor-Joining tree for the MyoD of several vertebrates. Bootstrap values are indicated in the nodes and the scale bar indicates the genetic distance.

production is found across the whole myotome. This results in a mosaic pattern of different fiber diameters, as seen in pacu skeletal muscle morphological analysis. Mosaic hyperplasia causes a large increase in fiber numbers during juvenile growth and is very important for commercial aquaculture species; this characteristic is not seen in small species (Rowlerson and Veggetti, 2001). In juvenile pacu, the mainly mosaic hyperplastic contribution was higher than hypertrophy in skeletal muscle growth.

In adult pacu, a majority of $>50\,\mu m$ diameter fibers denotes muscle fiber hypertrophy (Valente et al., 1999; Rowlerson and Veggetti, 2001). According to Zimmerman and Lowery (1999), the recruitment of new fibers during muscle growth stops when fish reach about 44% of their final size; after this muscle growth is mainly by hypertrophy. Although the commercially interesting size of the pacu is not fixed, our study showed that muscle fiber recruitment in the adult phase was lower than in the juvenile phase.

Hyperplasia and hypertrophy in fish muscle growth is dependent on the activation, proliferation, and differentiation of adult myoblast or myosatellite cells (Koumans and Akster, 1995; Johnston, 1999). These processes are regulated by the sequential expression of transcription factors known as myogenic regulatory factors (Watabe, 1999, 2001).

MRF expression levels play an essential role during myogenesis and are related to myoblast specification and differentiation, and regulate muscle development and growth in growing fish (Zhang et al., 2006). In flounder (*Paralichthys olivaceus*) MyoD expression was detected in precursor muscle cells during the initial phases of embryogenesis (Zhang et al., 2006). Johansen and Overturf (2005) showed continuous differential MRF (MyoD, Myf5, Myogenin and MRF4) expression in rainbow trout (*Oncorhyncus mykiss*) skeletal muscle during different growth phases. These authors inferred that differential MRF expression may be related to muscle growth mechanisms.

In our study MyoD mRNA level was significantly higher in juvenile than adult pacu. During early development and the juvenile stage, muscle growth occurs by intense recruitment of new muscle fibers from the proliferation of undifferentiated myogenic progenitor cells that express primary MRF, MyoD, and Myf5 (Rescan et al., 1994; Watabe, 2001; Megeney and Rudnicki, 1995). Myoblast proliferation is directly related to the hyperplastic process and both can be inferred by analyzing expression levels of MyoD and Myf5 (Johansen and Overturf, 2005). In our study, the high MyoD expression levels in juvenile fish can be associated to a predominant hyperplastic mechanism in muscle growth.

In adult *P. mesopotamicus*, muscle growth was mainly by hypertrophy. In this stage, myoblast proliferation and hyperplasia are not significant, with MyoD expression being smaller than in juvenile fish (Johansen and Overturf, 2005). This can explain the low MyoD expression in adult pacu compared to their juvenile counterparts.

Comparative analysis of pacu MyoD cDNA nucleotide sequence showed a close relationship for this gene in fish that were branched out in relation to amphibians, birds, and mammals. The analysis of the complete cDNA of MyoD of several representatives of the main vertebrate groups will clarify the evolutionary history of MyoD among vertebrates.

The expression of genes that control muscle growth is still unknown in South American fish species. The results from our study should provide a foundation for understanding the molecular control of skeletal muscle growth in economically important Brazilian species, with a view to improving production quality.

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