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IMPLICAÇÕES DO USO DE MARCADORES MOLECULARES PARA O TRANSPLANTE DE CÉLULAS GERMINATIVAS EM PEIXES

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IMPLICAÇÕES DO USO DE MARCADORES MOLECULARES PARA O TRANSPLANTE DE CÉLULAS GERMINATIVAS EM PEIXES [1]

Autora: Ana Carina Nogueira Vasconcelos
Orientador: Dr. Danilo P. Streit Jr.

Resumo: O transplante de células germinativas tem sido uma importante abordagem experimental para o estudo da preservação genética de espécies ameaçadas de extinção ou economicamente importantes. A técnica consiste na remoção das células germinativas indiferenciadas das gônadas do animal doador e na transferência das mesmas para a gônada de um indivíduo receptor. A fim de aumentar a eficiência da técnica, a identificação prévia das células germinativas a serem transplantadas torna-se preferível, visto que a cavidade que as receberá apresenta tamanho limitado. Sendo assim, é importante o desenvolvimento de marcadores moleculares que identifiquem precisamente as células a serem transplantadas na cavidade do individuo receptor, e os genes mais utilizados para esta finalidade são o *dead end* e o gene *vasa*, os quais são expressos apenas nas células da linhagem germinativa. Devido à importância do *Colossoma macropomum* (tambaqui) para a economia brasileira, esta espécie foi escolhida como uma espécie modelo de preservação para este estudo. Através do isolamento e sequenciamento dos genes *dead end* e *vasa,* desenvolvemos sondas de hibridização capazes de reconhecer as células onde estes genes são expressos e estudar o seu padrão de expressão nas gônadas. Ambos os genes apresentaram intensa expressão nos oócitos pré-vitelogênicos e fraca expressão em algumas espermatogônias. Pela primeira vez na literatura, diferentes isoformas causadas por *splicing* alternativo foram identificadas no gene *dead end.* A quantificação da expressão temporal dos diferentes transcritos mostrou que o padrão de expressão da sequência completa do gene teve uma tendência distintiva comparada ao padrão dos transcritos curtos, sugerindo que as diferentes isoformas desempenham papéis específicos e importantes para o desenvolvimento da linha germinativa nesta espécie.

Palavras-chave: células germinativas, expressão gênica, sequenciamento genético, tambaqui.

[1] Tese de Doutorado em Zootecnia - Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (108 p.) Março, 2018.

IMPLICATIONS OF THE USE OF MOLECULAR MARKERS FOR THE GERM CELLS TRANSPLANTATION IN FISH[2]

Author: Ana Carina Nogueira Vasconcelos
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Abstract: Germ cell transplantation has been an important experimental approach to the study of the genetic preservation of endangered or economically important species. The technique consists in removing undifferentiated germ cells from the donor animal's gonads and transferring them to the gonad of a recipient individual. In order to increase the efficiency of the technique, the prior identification of the germ cells to be transplanted becomes preferable, since the receiving cavity presents limited size. Therefore, it is important to develop molecular markers to precisely identify the cells to be implanted in the recipient cavity, and the genes most used for this purpose are the *dead end* and the *vasa* genes, which are expressed only in germline cells. Due to the importance of *Colossoma macropomum* (tambaqui) for the Brazilian economy, this species was chosen as a model species for preservation in this study. By isolating and sequencing the *dead end* and *vasa* genes, we developed hybridization probes capable of recognizing the cells where these genes are expressed and better studying their expression pattern in the gonads. Both genes presented intense expression in pre-vitellogenic oocytes and poor expression in some spermatogonia. For the first time in the literature, different isoforms caused by alternative splicing were identified in the *dead end* gene. Quantification of the temporal expression of the different transcripts showed that the expression pattern of the full-length sequence had a distinctive tendency compared to the short transcripts pattern, suggesting that the different isoforms play specific roles for the germline development in this species.

Keywords: gene expression, genetic sequencing, germ cells, tambaqui.

[2] Doctoral thesis in Animal Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (108 p.) March, 2018.

Sumário

Lista de Tabelas

Lista de Figuras

Lista de abreviaturas e símbolos

bp: Base pair
cDNA: Complementary DNA
DNA: Deoxyribonucleic acid
dnd: dead end
DSRM: Double-stranded RNA binding motif
Fw: Foward
ISH: *In situ* hybridization
miRNA: Micro ribonucleic acid
mRNA: Messenger ribonucleic acid
RACE: Rapid Amplification of cDNA Ends
RNA: Ribonucleic acid
RRM: RNA recognition motif
RT-PCR: Reverse Transcription–Polymerase Chain Reaction
Rv: Reverse

CAPÍTULO I

1. Introdução

Popularmente conhecida como tambaqui, a espécie amazônica *Colossoma macropomum* é uma das mais utilizadas em piscicultura no Brasil devido à facilidade na produção de alevinos e ao rápido crescimento (Marcuschi et al., 2010). O tambaqui é um peixe de grande porte e sua produção tem apresentado um crescimento exponencial nos últimos anos (Batista & Petrere Jr., 2003; Sánchez-Botero & Garcez, 2006; Sousa et al., 2008).

Apesar das populações ainda apresentarem adequada variabilidade genética na natureza (Fazzi-Gomes et al., 2017), a fragmentação e/ou destruição do habitat natural (Araújo-Lima & Ruffino, 2004; Sánchez-Botero & Garcez, 2006), juntamente com as alterações climáticas e sobrepesca (Sánchez-Botero & Garcez, 2006; Sousa et al., 2008)), podem provocar o desaparecimento de indivíduos menos adaptados, diminuindo assim, o tamanho populacional da espécie.

Para possibilitar a diversidade genética equivalente ao das populações naturais, a produção de indivíduos jovens em cativeiro deve ser efetuada utilizando uma quantidade significativa de reprodutores. Entretanto, algumas variáveis impedem o uso de um elevado número de indivíduos, como a assincronia no tempo de desova e os custos para a manutenção dos animais.

Há alguns anos, uma técnica de transplante interespecífico de células germinativas do tipo A vem sendo estudada com o propósito de solucionar problemas de baixa variabilidade genética de algumas espécies em cativeiro e dificuldade de manutenção das matrizes (Takeuchi et al., 2004; Farlora et al., 2014; Sato et al., 2014). Esta técnica permite que as espermatogônias do doador colonizem a cavidade celomática do receptor e se diferenciem em ovos ou espermatozoides (Okutsu et al., 2006a; Okutsu et al., 2007; Takeuchi et al., 2009; Yazawa et al., 2010). E caso as células de mais de um doador sejam misturadas, um único indivíduo receptor produzirá gametas de animais diferentes (Sato et al., 2014), aumentando assim, a variabilidade genética dentro da população.

O sucesso obtido nas espécies yellowtail (Morita et al., 2012), tilápia (Farlora et al., 2014), pufferfish (Hamasaki et al., 2017), zebrafish (Li et al., 2017), medaka (Seki et al., 2017) e roncadeira-japonesa (Yoshikawa et al., 2017) viabiliza a aplicação desta biotecnologia como uma ferramenta de preservação de espécies ameaçadas de extinção e aumento da variabilidade genética em populações de interesse ambiental ou econômico.

Para identificar as células germinativas após a dissociação do tecido gonadal e analisar o comportamento das mesmas, o uso de marcadores moleculares é aconselhável. Como os genes *dead end* (Weidinger et al., 2003) e *vasa* (Raz, 2000) são especificamente expressos nas células da linhagem germinativa, o estudo de ambos tornou-se uma ferramenta importante para a identificação das células que serão transplantadas.

Com o objetivo geral de fornecer subsídios para a preservação de espécies de peixes econômica e ecologicamente importantes no Brasil, nesta tese serão abordados os principais fatores que colocam em risco as espécies brasileiras e o estudo dos genes *dead end (dnd)* e *vasa* da espécie *Colossoma*

macropomum. Para identificar as células germinativas desta espécie, isolamos e caracterizamos os genes *dnd* e *vasa* do tambaqui e avaliamos o potencial dos mesmos para serem utilizados como marcadores moleculares.

No primeiro capítulo, é apresentada uma revisão bibliográfica sobre os peixes teleósteos e os riscos de extinção das espécies, gametogênese, transplante de células germinativas e uma descrição sobre a importância dos genes *dead end* e *vasa* em diferentes espécies. No segundo capítulo, são apresentadas as técnicas de isolamento e sequenciamento do gene *vasa*, considerando o mesmo como um bom marcador molecular para os oócitos de tambaqui. O terceiro capítulo contém informações sobre o sequenciamento do gene *dead end* e a descoberta de três transcritos do gene encontrados em machos e fêmeas de tambaqui. Em seguida, serão apresentadas as considerações finais da tese.

2. Revisão bibliográfica

2.1. Peixes teleósteos

Os Osteichthyes, popularmente conhecidos como peixes ósseos, representam um diversificado grupo que possui esqueleto composto basicamente de tecido ósseo. Esta superclasse é a maior classe de vertebrados existentes, a qual consiste de mais de 61.000 espécies (Nelson et al, 2016). Atualmente é dividida em Sarcopterygii e Actinopterygii, sendo que os peixes teleósteos estão inseridos nesta última (Figura 1).

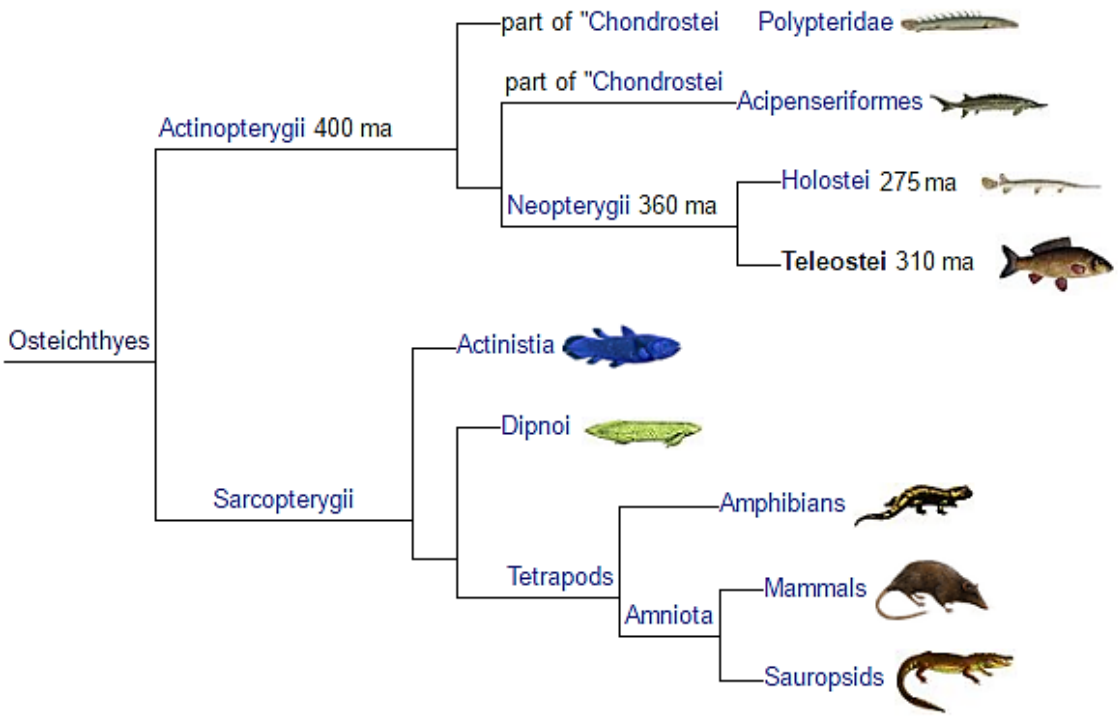

Figura 1. Dendograma relativo entre os peixes teleósteos e os demais grupos de vertebrados, mostrando também os tetrápodes que evoluíram a partir dos grupos de peixes. Adaptado de Near et al., (2012).

A infraclasse Teleostei surgiu no período Triássico e corresponde a aproximadamente 96% de todos os peixes conhecidos atualmente (Figura 2). Os seus membros formam um grupo monofilético e estão distribuídos em cerca de 63 ordens, 469 famílias e mais de 29.000 espécies, das quais 10% foram descobertas na última década (Nelson et al., 2016).

Apesar dos teleósteos adotarem diferentes estratégias reprodutivas, a maioria apresenta fertilização externa (ovulíparos). Uma parcela desta infraclasse é hermafrodita, começando a vida como fêmea e fazendo a transição para macho em algum momento, e apenas uma pequena porcentagem é vivípara (Helfman et al., 2009).

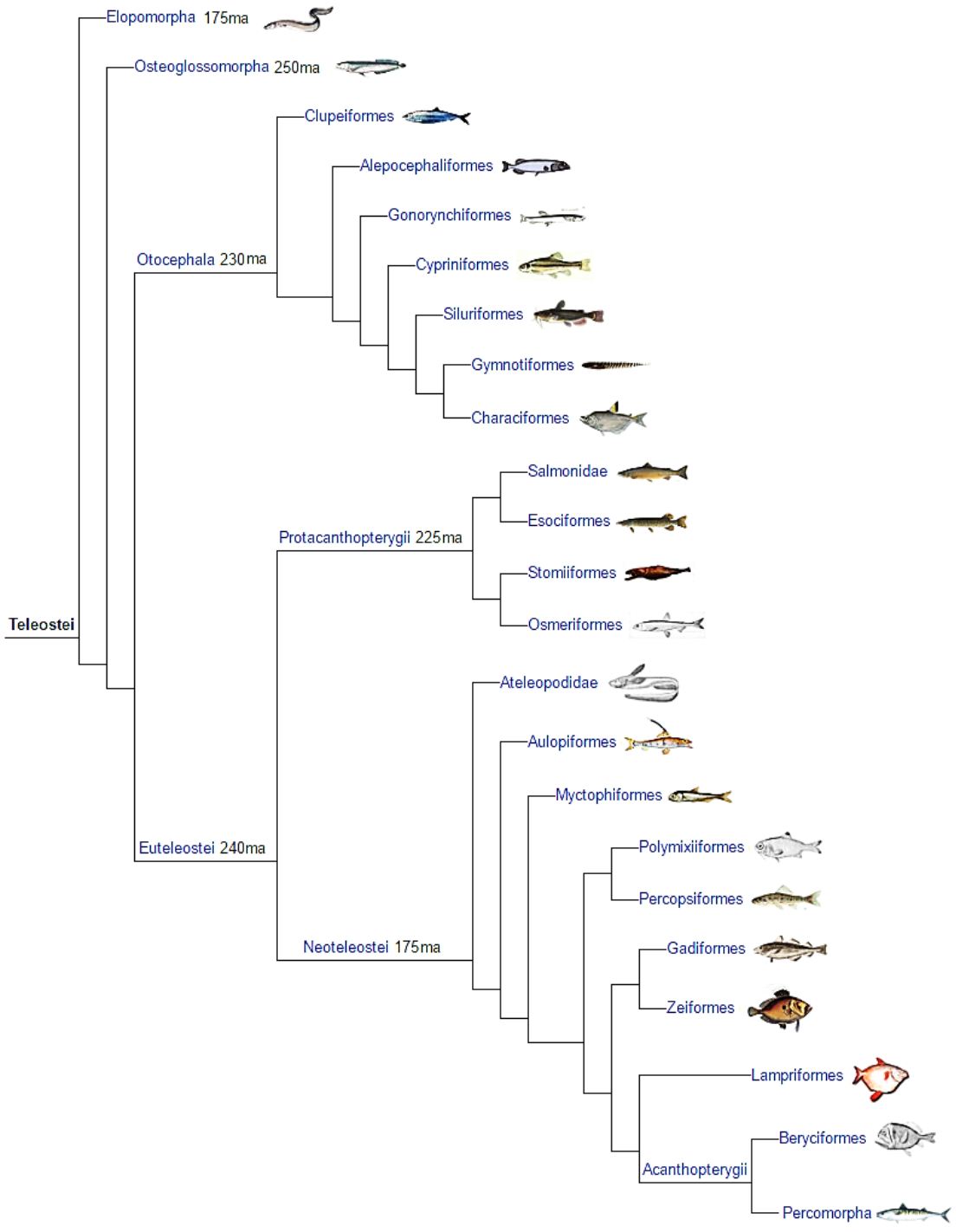

Figura 2. Cladograma mostrando as relações internas dentro da infraclasse Teleostei. Adaptado a partir de Near et al., (2012).

Até o presente momento, os animais exclusivamente dulciaquícolas estão restritos à superordem Osteoglossomorpha, sendo os demais grupos representados por peixes que vivem em ambientes marinhos e de água doce (Nelson, 1994).

Atualmente, o homem interfere decisivamente no processo natural de extinção das espécies através da destruição e/ou alteração de habitats, introdução de espécies exóticas, exploração dos recursos naturais e sobrepesca. De acordo com a Portaria do Ministério do Meio Ambiente n°445/2014, 475 espécies de peixes e invertebrados aquáticos precisam de proteção ambiental e não podem mais ser capturadas, transportadas, manejadas, armazenadas ou comercializadas. O número de animais em perigo de extinção cresce consideravelmente em todo o mundo devido aos desastres naturais, mudanças climáticas e à incapacidade de adaptação a novas condições impostas para sobreviverem.

2.2. Influência dos impactos ambientais

O aumento da temperatura média dos oceanos e da atmosfera da Terra é chamado de aquecimento global e é resultante, principalmente, do acúmulo de gases do efeito estufa. Esta alteração representa uma grande ameaça ao planeta (IPCC, 2014) e estudos comprovam que as mudanças climáticas reduzem a biodiversidade mundial (Ficke et al., 2007; Chown et al., 2010).

A América do Sul é uma das regiões mais vulneráveis às mudanças climáticas devido à degradação florestal e ao enfraquecimento do ciclo hidrológico (Hirota et al., 2011). Desta forma, poderá ter sua temperatura média aumentada drasticamente produzindo efeitos catastróficos em diversas regiões do continente, principalmente na Amazônia e no Nordeste brasileiro (Nobre et al., 2007; Hirota et al., 2010).

Inúmeros cenários criados pelo IPCC sugerem que a temperatura média na Amazônia aumentará mais de 3°C até o ano de 2100, o que reduziria em até 40% a cobertura vegetal de alguns territórios, ameaçando a fauna e a flora da região que possui a maior biodiversidade do planeta (IPCC, 2007; Nobre et al., 2007). O clima mais seco afetará o ambiente aquático e provocará alterações nos ecossistemas e comunidades de organismos (Portner & Farrell, 2008; Gamito et al., 2013).

Estudos em todos os níveis de organização biológica dos peixes revelam que os impactos ambientais afetam a distribuição geográfica (Perry et al., 2005), o comportamento (Abrahams et al., 2007), a fisiologia (Del Toro-Silva et al., 2008), a estrutura esquelética (Booth et al., 2014; Lopes, 2017) e a expressão de genes (Clark et al., 2011).

A sobrepesca é outro fator preocupante em relação ao desaparecimento de espécies. Somada às alterações climáticas (Ficke et al., 2007; Chown et al., 2010), a retirada de indivíduos menores e até mesmo antes da reprodução, diminui o estoque natural e pode reduzir a diversidade genética ao longo do tempo através da deriva genética estocástica (Willi et al., 2006). Com um menor fluxo gênico, a população pode não ser capaz de se adaptar a novas pressões seletivas.

Na natureza, os ecossistemas são organizados de maneira sustentável e todos os organismos são responsáveis por alguma ação preponderante para o seu equilíbrio. Se alguma espécie reduzir consideravelmente o seu tamanho populacional, todo o sistema poderá entrar em desequilíbrio, causando uma reação em cadeia e impactando o meio

ambiente. Desta forma, o estudo de alternativas de preservação genética das espécies torna-se importante para minimizar os impactos negativos causados pela redução da variabilidade genética.

2.3. Transplante de células germinativas

O transplante de células germinativas tem se tornado uma valiosa abordagem experimental para a preservação de espécies ameaçadas de extinção (Orwig & Schlatt, 2005; Lacerda et al., 2006; Higuchi et al., 2011; Yoshizaki et al., 2011; Morita et al., 2012), bem como o estudo da espermatogênese (Dobrinski, 2006; Hill & Dobrinski, 2006), preservação e manipulação da fertilidade (Dobrinski, 2008) e pesquisas na área de produção de animais transgênicos (Nagano et al., 2001; McLean, 2005; Kanatsu-Shinohara et al., 2008; Wong et al., 2011). Esta técnica consiste na remoção das células germinativas da gônada de um indivíduo doador e na transferência das mesmas para a cavidade celomática de larvas recém-eclodidas ou para a gônada de um receptor infértil, fazendo com que estas células se desenvolvam e formem gametas maduros com características genéticas do doador.

A produção de teleósteos quimeras através do transplante celular foi desenvolvida em 1992 (Lin et al., 1992) e em 1994, esta metodologia começou a ser estudada em mamíferos (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994). Estudos posteriores mostraram um importante avanço científico com o sucesso na produção de gametas gerados a partir do transplante xenogênico (interespecífico) de espermatogônias-tronco de rato para camundongo e de hamster para camundongo (Clouthier et al., 1996; Ogawa et al., 1999). Estudos recentes com transplante singênico (intraespecífico) mostraram resultados satisfatórios em yellowtail (Morita et al., 2012), tilápia (Farlora et al., 2014), truta arco-íris (Takeuchi et al., 2003, 2004, 2009; Lee et al., 2013, 2015, 2016a, 2016b), truta asiática (Lee & Yoshizaki, 2016), medaka (Seki et al., 2017), zebrafish (Li et al., 2017), pufferfish (Hamasaki et al., 2017) e roncadeira-japonesa (Takeuchi et al., 2009; Yoshikawa et al., 2017). Ainda que as células germinativas dos animais doadores sejam capazes de colonizar as gônadas de um animal receptor, a eficiência do transplante depende da competição entre as células transplantadas e as endógenas (Shinohara et al., 2001, 2002). Desta forma, o receptor ideal deve conter células somáticas normais e a gametogênese endógena ausente ou depletada (Brinster et al., 2003), cuja depleção nos peixes pode ser provocada pela droga quimioterápica busulfan (1,4-dimetanosulfonoxibutano) (Lacerda, 2006; Lacerda et al., 2008, 2010; Majhi et al., 2009; Nóbrega et al., 2010), , uso de triploides estéreis (Okutsu et al., 2007) e técnicas de knockdown (Yoshizaki et al., 2016).

Em peixes, a realização do transplante utilizando células germinativas criopreservadas (Orwig & Schlatt, 2005; Okutsu et al., 2006b; Lee et al., 2013; Lee et al., 2016) tem mostrado avanços desta técnica e a possibilidade de preservação das linhagens de células germinativas de animais de alto valor zootécnico, espécies em extinção e animais experimentais valiosos. Ampliando ainda mais este cenário, Shikina et al. (2008) demonstraram a capacidade de expansão de espermatogônias do tipo A em

um sistema *in vitro*, antes das mesmas serem transplantadas nos indivíduos receptores.

2.3.1. Perspectivas do transplante de espermatogônias

Particularmente em teleósteos, o uso do transplante singênico ou xenogênico cria um cenário favorável na área de biotecnologia, conservação genética e produção em aquicultura (Lacerda et al., 2006). A técnica possibilita o estudo da biologia da reprodução e dos mecanismos de regulação da gametogênese (Schulz et al., 2010), preservação de espécies ameaçadas de extinção ou economicamente importantes (Okutsu et al., 2006b; Yazawa et al., 2013), conservação do material genético de animais melhorados, viabiliza o aumento da diversidade genética em curto prazo (Lacerda et al., 2006) e análise *in vivo* do comportamento e interação das células que participam ativamente da gametogênese (Takeuchi et al., 2003). A maior importância desta técnica está nas possibilidades de investigação da biologia das células germinativas, visto que o transplante celular proporciona uma análise da gametogênese. Embora o transplante de espermatogônias apresente um enorme potencial teórico, a eficiência desta técnica ainda é relativamente baixa, o que aponta para a necessidade de estudos mais precisos em diferentes espécies (Griswold et al., 2001; Aponte et al., 2005; McLean, 2005; Ehmcke et al., 2006; Nóbrega et al., 2010). Estudar e entender o processo de gametogênese é fundamental para preservar uma espécie de interesse através da técnica de transplante celular, pois é necessário conhecer as características das células que serão utilizadas para colonizar a cavidade celomática do animal receptor.

2.4. Gametogênese nos animais

O processo gametogênico é o evento responsável pela produção dos gametas nos animais de reprodução sexuada. Este ciclo ocorre nas gônadas e tem a meiose como evento fundamental, a qual reduz pela metade a quantidade de cromossomos das células, originando células haploides. A diploidia característica de cada espécie é reestabelecida após a fecundação, quando ocorre a fusão dos dois gametas haploides provindos do macho e da fêmea.

2.4.1. Espermatogênese

No interior do túbulo seminífero, o processo espermatogênico ocorre de maneira cíclica e altamente organizada, dando origem aos espermatozoides a partir da diferenciação de espermatogônias diploides (Figura 3A). Baseado nas características morfofuncionais, a espermatogênese pode ser dividida em três fases (Figura 3B). Na fase proliferativa ou espermatogonial, as espermatogônias se dividem rápida e sucessivamente através da divisão mitótica, e estas células terão seu material genético duplicado, recombinado e segregado durante a fase meiótica ou espermatocitária. Esta segunda fase é importante para a diversidade genética entre membros de uma espécie e prepara as células para a fase de diferenciação ou espermiogênica, quando as espermátides passam por transformações morfogenéticas, que culminam na formação dos espermatozoides (Russell et al., 1990; Sharpe, 1994). Após as

três etapas, os espermatozoides serão liberados no lúmen do túbulo seminífero, e a maturação espermática fará com que o gameta, morfologicamente preparado, torna-se funcionalmente apto para fertilizar o oócito (Yaffe, 1997; Schulz & Miura, 2002; Miura & Miura, 2003).

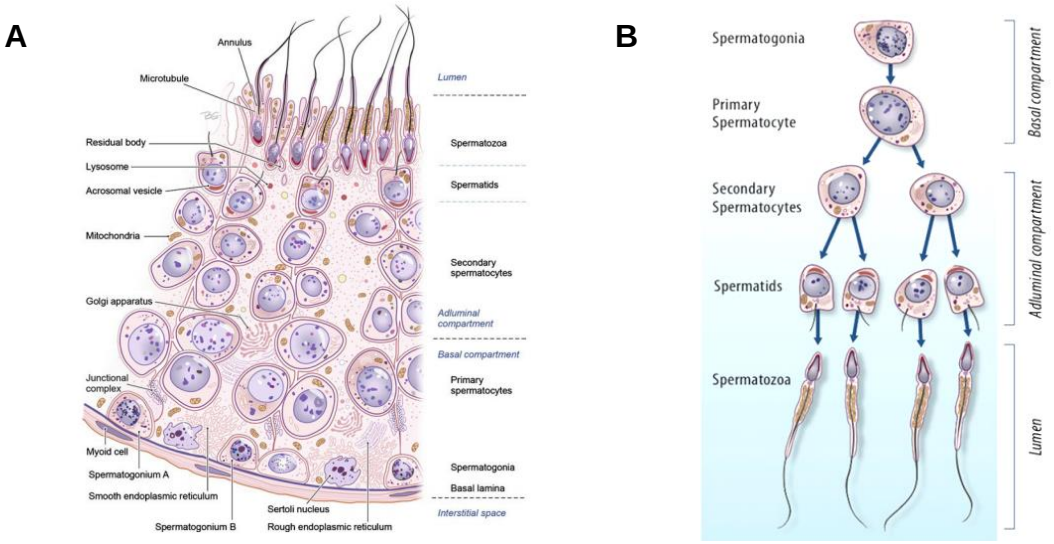

Figura 3. Representação das fases da espermatogênese. (A) Seção do epitélio germinal no túbulo seminífero, mostrando o local onde cada etapa da espermatogênese acontece. (B) Representação simplificada dos principais eventos da espermatogênese, durante a qual as células germinativas sofrem divisão mitótica e meiótica para reduzir o conteúdo cromossômico. Fonte: Sharma & Agarwal, 2011 (reimpresso com permissão, Cleveland Clinic Center for Medical Art & Photography).

2.4.2. Espermatogênese nos peixes teleósteos

O processo espermatogênico nos peixes ocorre no interior de estruturas denominadas espermatocistos, ou cistos, que se formam a partir do envolvimento de uma espermatogônia primária ou do tipo A, pelos prolongamentos das células de Sertoli (Grier, 1993; Pudney, 1993 e 1995; Schulz et al., 2010). Além de fornecer suporte hormonal, mecânico e nutricional às células germinativas, as células de Sertoli fagocitam os corpos residuais, resultantes dos processos de morte celular programada e eliminação de citoplasma durante a formação espermática (Billard, 1970; Schulz & Nóbrega, 2011). As células germinativas derivadas desta espermatogônia continuam envolvidas por um número variado de células de Sertoli e sofrem divisões sincronizadas (Vilela et al., 2003). Portanto, diferentemente de mamíferos (Russell et al., 1990), as células de Sertoli normalmente estão em contato com apenas um grupo específico de célula germinativa, as quais evoluem ao longo do processo espermatogênico (Nóbrega et al., 2009; Schulz et al., 2010) (Figura 4).

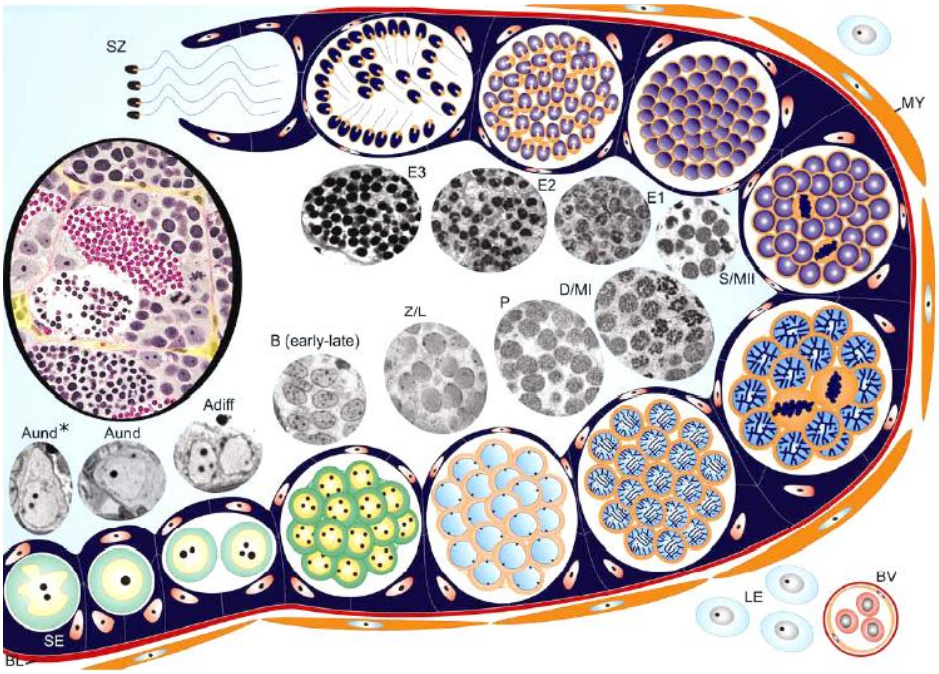

Figura 4. Desenho esquemático da espermatogênese dos peixes mostrando as células de Sertoli em contato com apenas um tipo específico de célula ao longo da espermatogênese. Legendas: células de Sertoli (SE); lâmina basal (BL); células peritubulares mióides (MY), células de Leydig (LE), espematogônia (SG); espermatócito (SC); espermátide arredondada (RST); espermátide alongada (EST); espermatogônia do tipo A indiferenciada (Aund); espermatogônia do tipo A diferenciada (Adiff); espermatogônia do tipo B (B early-late); espermatócitos primários em leptóteno/zigóteno (L/Z), paquíteno (P), diplóteno/metáfase I (D/MI); espermatócitos secundários/metáfase II (S/MII); espermátides iniciais (E1); intermediárias (E2); finais (E3); espermatozoides (SZ); e vasos sanguíneos (BV). Fonte: Schulz et al., 2010.

2.4.3. Oogênese

As células reprodutoras femininas, os oócitos, são formadas a partir de um processo denominado oogênese (Figura 5), o qual é dividido em três etapas. Durante o processo de proliferação ou multiplicação, uma série de divisões mitóticas produz um grande número de oogônias. As sobreviventes transformam-se em células maiores durante o período de crescimento, acumulando substâncias nutritivas, e são denominadas oócitos primários ou oócitos I, os quais iniciam a meiose. Este processo é interrompido na prófase I e as células só terminam sua primeira divisão quando o indivíduo atinge a maturidade. As duas células filhas geradas têm tamanho diferentes, sendo que o oócito secundário recebe a maior parte do citoplasma e o primeiro corpúsculo polar praticamente não o recebe. Este oócito, que será lançado no momento da ovulação, começa a sofrer a segunda divisão da meiose, mas o processo é inibido na metáfase II. Caso não haja fecundação, a célula se degenera em aproximadamente um dia após a sua liberação. Entretanto, se houver o encontro do espermatozoide com o oócito II, a meiose continua e o oócito II

divide-se em duas células desiguais, o segundo corpúsculo polar que irá se degenerar, e ovo que é a união dos gametas feminino e masculino.

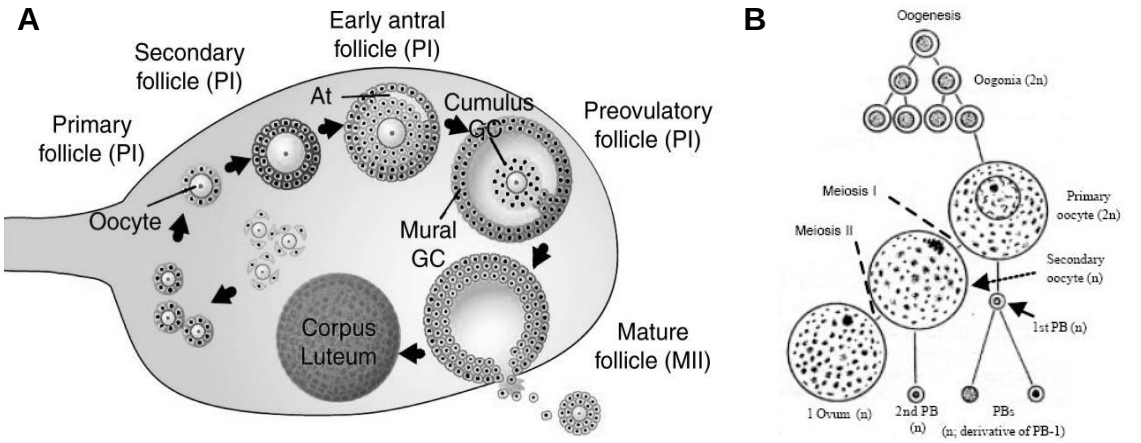

Figura 5. Oogênese. (A) Desenho esquemático das etapas do desenvolvimento dos oócitos e progressão meiótica. Legendas: folículo primário (P1); células da granulosa (GC); antro (At); oócito secundário (MII). Fonte: Von Stetina & Orr-Weaver, 2011. (B) Representação simplificada dos principais eventos da oogênese. Legendas: corpúsculo polar (PB); primeiro corpúsculo polar (PB-1). Fonte: Rahman et al., 2008.

2.4.4. Oogênese nos peixes teleósteos

O desenvolvimento ovariano em peixes é separado em três categorias (Wallace and Selman, 1981): (a) síncrono, quando todos os oócitos desenvolvem e são liberados ao mesmo tempo, (b) síncrono em grupo, quando pelo menos duas populações de oócitos podem ser reconhecidas no ovário durante a estação reprodutiva, e (c) assíncrono, quando os oócitos de todos os estágios de desenvolvimento estão presentes sem uma população dominante.

O desenvolvimento dos oócitos (Figura 6) e o momento da desova podem diferir entre espécies, porém alguns estudos mostram diversas características semelhantes na formação dos oócitos maduros. A oogênese descrita anteriormente também acontece na maioria dos peixes teleósteos, mas vale ressaltar que a divisão em diferentes estágios ou etapas é artificial, visto que o desenvolvimento de oócitos é um processo bastante dinâmico e difícil de identificar o início ou o final de cada etapa (Lubzens et al., 2010). Uma importante diferença em comparação aos mamíferos é a proliferação contínua das oogônias nos peixes, renovando os estoques de oócitos e folículos jovens ao longo da vida (Tokarz, 1978).

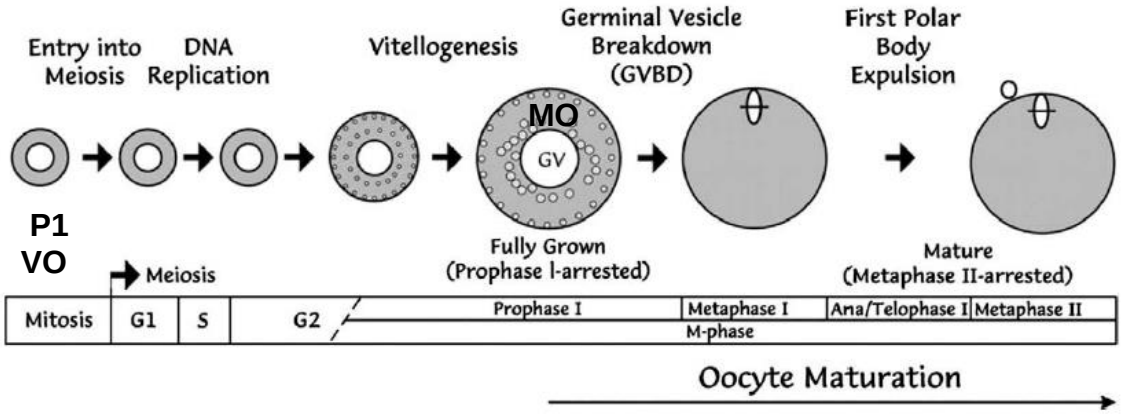

Figura 6. Descrição esquemática dos estádios de desenvolvimento dos oócitos em peixes teleósteos. Legenda: oócitos primários (P1), oócitos vitelogênicos (VO) e maduros (MO). Fonte: Lubzens et al., 2010.

2.5. Gene *dead end*

A identificação das células germinativas entre células testiculares e ovarianas é um passo crítico para o sucesso do transplante de espermatogônias e oogônias, e o desenvolvimento de marcadores moleculares torna-se um importante aliado na distinção das PGCs após a dissociação dos tecidos gonadais.

Identificado pela primeira vez em zebrafish (Weidinger et al., 2003), o gene *dead end* (*dnd*) é um componente do germoplasma e codifica uma proteína de ligação crucial para a migração e sobrevivência de células primordiais. Esta proteína é localizada em grânulos perinucleares dentro das PGCs, e diversos estudos de *knockdown* confirmaram a importância do *dnd* para o desenvolvimento adequado das gônadas em peixes teleósteos (Weidinger et al., 2003; Horvay et al., 2006; Kurokawa et al. , 2007; Siegfried e Nusslein-Volhard, 2008; Skugor et al., 2013). Embora o gene seja expresso de maneira específica na linhagem germinativa durante a embriogênese de rã, galinha e rato (Weidinger et al., 2003), em organismos adultos, a expressão do *dnd* parece ser dependente do sexo do indivíduo. No rato, a expressão do *dnd1α* é exclusiva em células germinativas de testículos adultos (Bhattacharya et al., 2007), enquanto que em rãs a transcrição do gene é encontrada apenas no ovário (Horvay et al., 2006). O padrão de expressão do *dnd* em adultos de medaka (Liu et al., 2009) e turbot (Lin et al., 2013) ocorre em células germinativas de ambos os sexos.

O fato de este gene ser expresso apenas nas células germinativas faz com que o *dead end* seja um potencial marcador molecular para as PGCs. O seu padrão de expressão nas gônadas de cada espécie pode ser verificado através do sequenciamento genético e desenvolvimento de sondas capazes de reconhecer os locais específicos de expressão deste gene.

2.6. Gene *vasa*

Identificado pela primeira vez na espécie *Drosophila melanogaster* (Raz, 2000), o gene *vasa* é conhecido por se ligar de forma específica a sequências de RNA, sendo essencial para o desenvolvimento de células

germinativas e determinação da função das mesmas. Recentemente, foi descoberto que esta proteína também exerce uma função nas células-tronco multipotentes, embora o envolvimento exato ainda seja desconhecido (Gustafson and Wessel, 2010).

O gene *vasa* é bastante conservado entre vertebrados e invertebrados e é essencial durante a gametogênese e embriogênese. Na maioria das espécies, este gene é expresso exclusivamente nas células germinativas (Olsen et al., 1997; Yoon et al., 1997; Yoshizaki et al., 2000; Kobayashi et al., 2000; Shinomiya et al., 2000; Knaut et al., 2000; Xu et al., 2005; Saito et al., 2004; Cao et al., 2012), tornando-se um importante candidato para a identificação deste tipo celular. A expressão do gene *vasa* pode ser distinta entre as espécies, podendo ocorrer em células mitóticas e meióticas de ambos os sexos do Kinguio (Xu et al., 2005) e medaka (Xu et al, 2009), nos oócitos vitelogênicos da dourada (Cardinali et al., 2004), ou até mesmo, em ovos infertilizados do robalo (Blazquez et al., 2011).

2.7. Tambaqui (*Colossoma macropomum*)

Popularmente conhecido como tambaqui, o *Colossoma macropomum* (Figura 7) é um peixe teleósteo migrador pertencente à família Characidae (Jegú, 2003) e destaca-se pelo grande potencial de cultivo em cativeiro. Esta espécie possui hábito alimentar diversificado (Honda, 1974; Goulding & Carvalho, 1982), rápido crescimento em águas ácidas (Aride et al., 2007), resistência à hipóxia (Baldisserotto, 2009) e à ação tóxica da amônia (Ismiño-Orbe, 1997), e é bastante apreciada por comunidades tradicionais na Amazônia (Gomes et al., 2010).

Figura 7. Tambaqui (*Colossoma macropomum).* Fonte: International Fish Association.

A espécie é nativa das bacias dos rios sul-americanos, Amazonas e Orinoco, podendo ser encontrada em diferentes áreas do continente (Figura 8). A ocorrência fora da área original se deve à alta resistência dos indivíduos, à alta frequência de escape nas fazendas produtoras de tambaqui (Lopes et al., 2017) e ao seu comportamento migratório, os quais favorecem o fluxo genético entre os sistemas hidrográficos (Fazzi-Gomes et al., 2017).

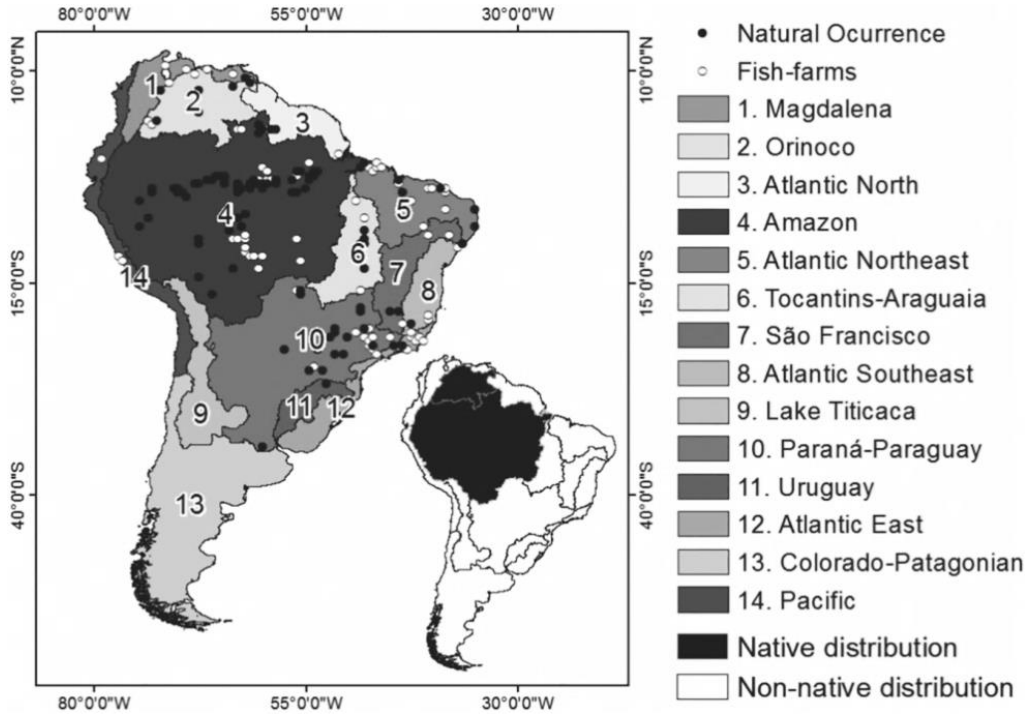

Figura 8. Ocorrência do *Colossoma macropomum* na América do Sul. O mapa menor representa a área de distribuição natural da espécie. Fonte: Lopes et al., 2017.

O tambaqui é o segundo maior peixe de escama da América do Sul (Gomes et al., 2010) e tem sido explorado pela pesca comercial na Amazônia desde o século XIX. Na década de 1980, a população do tambaqui declinou na Amazônia devido à captura excessiva, e o IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) colocou a espécie na lista de animais protegidos durante o período de defeso (1º de novembro a 28 de fevereiro) (Boischio, 1992; Val & Honczark, 1995; IBAMA, 2003). Durante este período, que coincide com a reprodução da espécie, a pesca fica proibida na tentativa de recuperar os estoques. Em 2003, o Instituto também estabeleceu restrições para pesca em qualquer época do ano (Portarias 65 e 67 de 30/10/2003) e o comprimento total mínimo para captura e comercialização do tambaqui passou a ser 55 cm (Batista et al., 2004).

Com o passar dos anos, o tamanho dos indivíduos selvagens reduziu drasticamente e dentre os animais capturados nos anos de 2007 e 2008, apenas 1% encontrava-se no tamanho acima do limite permitido pelo IBAMA (Sousa et al., 2008; Garcez & Freitas, 2010). Desta forma, fica evidente que a alta taxa de exploração do tambaqui na região Amazônica está acima do rendimento máximo sustentável, indicando uma sobrepesca dos estoques remanescentes (Isaac & Ruffino, 1996; Batista & Petrere Jr., 2003; Araújo-Lima & Ruffino, 2004; Sánchez-Botero & Garcez, 2006; Sousa et al., 2008). A queda no abastecimento dos mercados, juntamente com a contínua redução do tamanho do peixe capturado, aumentou a demanda pelo tambaqui e os investimentos em produção em cativeiro e no cultivo da espécie.

Em cativeiro, o tambaqui atinge idade reprodutiva entre 4 e 5 anos, com tamanho aproximado de 55 cm, e as fêmeas podem desovar até duas vezes por ano (Streit Jr. et al., 2012). A idade reprodutiva é dependente das condições de manejo e cultivo, e alguns animais podem estar aptos para reproduzir antes da idade citada. Os indivíduos são ovulíparos, de desova total e a eclosão das larvas ocorre entre 20 e 25 horas após a fertilização, quando incubadas a uma temperatura média de 25°C.

Embora as populações de tambaqui ainda apresentem níveis satisfatórios de diversidade genética (Fazzi-Gomes et al., 2017), a redução dos estoques selvagens pode limitar o fluxo gênico entre os indivíduos e reduzir drasticamente a variabilidade genética do tambaqui em médio prazo (Figura 9).

Figura 9. Fatores que influenciam direta ou indiretamente na redução da variabilidade genética do tambaqui.

3. Hipóteses e objetivos

A hipótese central do trabalho propõe a possibilidade de isolamento dos genes *dead end* e *vasa* através da clonagem molecular, permitindo o estudo da expressão gênica nos tecidos de tambaqui. Neste sentido, o objetivo geral foi isolar e sequenciar os genes para estudo de suas expressões nas gônadas de indivíduos de idades distintas, e desenvolver sondas moleculares capazes de identificar as células germinativas primordiais.

Os objetivos específicos foram:

1. Clonar os genes *dead end* e *vasa*, utilizando a transformação e multiplicação bacteriana.
2. Sequenciar os genes para a obtenção das fases de leitura aberta que são compreendidas entre os códons de iniciação e terminação.
3. Utilizar os genes *dead end* e *vasa* como marcadores moleculares de células germinativas.

CAPÍTULO II

Cloning, characterization and expression of *vasa* gene in the teleost fish *Colossoma macropomum*

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Cloning, characterization and expression of *vasa* gene in teleost fish *Colossoma macropomum*

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Abstract

The amazon species tambaqui *Colossoma macropomum* is a migratory fish with great potential for cultivation. The species is the second largest scaled fish in South America and is the native species most produced in Brazil. In consequence to the overfishing, the native fish stocks reduced considerably throughout the Amazon and large investments have been made to meet the demand for the species. Despite the populations in captivity still show satisfactory levels of genetic diversity, the reduced fish stock can prevent the genetic flow among individuals. In order to preserve the genetic resources possessing enough genetic diversity, the germ cell cryopreservation and transplantation have been studied in many fish species. To trace the behavior of the germ cell during their manipulation researches, a molecular marker to identify these cells is required and the *vasa* gene is a promising candidate since, in most species, research revealed a specific expression of the gene in the germ cells. Vasa is an RNA binding protein that encodes an ATP-dependent RNA helicase belonging to the DEAD-box family. It was first identified in *Drosophila* and known to be essential for germ cell development. In this study, the full sequence of *vasa* cDNA homolog from tambaqui was isolated and characterized, showing an open reading frame of 2,010 bp encoding 669 amino acids. The putative protein was shown to contain eight conserved motifs of the DEAD-box protein family and high similarity to *vasa* homologs of other species. The tambaqui *vasa* (*tvasa*) mRNA expression was specific to the gonad and the *in situ* hybridization showed signal only in oocytes and spermatogonia. These results suggested that *tvasa* can be used as a germ cells marker of this species.

Keywords: germ cells, molecular biology, sequencing, tambaqui

1. Introduction

Tambaqui (*Colossoma macropomum*) is the second largest scaled fish in South America and is the native fish most produced in Brazil. This migratory species is recognized for its great potential for captive cultivation due to its diversified food habit (Honda, 1974; Goulding & Carvalho, 1982), rapid growth in acidic water (Aride et al., 2007) andresistance to hypoxia (Baldisserotto, 2009). Since the species has been exploited by commercial fishing in the Amazon since the 19th century, fish stocks reduced considerably throughout the Amazon (Batista & Petrere Jr., 2003) and large investments have been made to enable captive cultivation and production, in order to meet the demand for the species.

Although tambaqui populations still show satisfactory levels of genetic diversity (Fazzi-Gomes et al., 2017), the reduction of the fish stock can limit gene flow among individuals and greatly reduce the wild population. Genetic diversity equivalent to that of natural populations can be achieved through the production of embryos in captivity using a sufficient number of breeding herds. However, the use of a high number of individuals, high costs in keeping the animals and spawning time asynchrony prevent this production.

In order to preserve the genetic resources semi-permanently, germ cell cryopreservation and its transplantation was developed and has been extensively studied today (Yoshizaki et al., 2011; Lee et al., 2013; 2015; 2016a; 2016b, 2016c). Since cryopreservation of fish eggs has not been possible due to its large size and high yolk and lipid contents, cryopreservation of immature germ cells can be an only method for long-term preservation of fish genetic resources (Yoshizaki et al., 2011). Recently, Lee et al (2013; 2015; 2016b) successfully developed a method to cryopreserve whole gonads and retrieve live germ cells. Further, the frozen and thawed germ cells were successfully converted to functional eggs and sperm viatheir transplantation into recipient fish (Lee et al., 2013). This transplantation technique consists of isolating germ cells from donor testes and injecting them into the peritoneal cavity of infertile recipients, giving rise to oocytes or spermatozoa, depending on the sex of the recipients (Okutsu et al., 2006a; Okutsu et al., 2007). Noticeably, if cells from more than one donor individual are mixed and used for the transplantation, a single recipient individual

produces gametes derived from different donor individuals (Sato et al., 2014), thereby increasing genetic variability within the population.

The success obtained in a variety of species (Morita et al., 2012;, Farlora et al., 2014; Seki et al., 2017; Li et al., 2017; Hamasaki et al., 2017; Yoshikawa et al., 2017) has made possible the application of this biotechnology as a tool to repopulate wide range of endangered species.

To identify the cells after dissociation of the gonadal tissue and analyze the behavior of the germ cells, use of molecular markers is preferable. *vasa* gene is specifically expressed in germ cells of a wide range of animals from insects to mammals (Raz, 2000) and it is essential for germ cell formation, differentiation and maintenance (Lasko & Ashburner, 1988; Tanaka et al., 2000; Hartung et al., 2014). Here, we isolated and characterized tambaqui *vasa* (*tvasa*) cDNA and evaluated its use as a molecular marker for germ-line cells.

2. Materials and methods

2.1. RNA isolation

Fragments of ovary, testis, heart, brain, gill, liver, muscle, kidney, intestine and stomach were collected from 6, 8, 16, 26 and 30 months-old tambaqui. Total RNA was extracted from all tissues using a QuickPrep Total RNA Extraction Kit (Amersham Pharmacia Biotech). The quality of the isolated RNA was examined by spectrophotometer and 0.7% denaturing gel electrophoresis.

2.2. Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Two micrograms of total RNA was used for cDNA synthesis. First strand cDNA was synthesized by means of Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech) with the adapter-oligo dT primer (5'-CTGATCTAGAGGTACCGGATCC-oligo dT- 3'). RT-PCR was performed with degenerate primers (Tq*vasa*-forward: 5'-CCMGSAAGTTYGCHTWTGGGACHTGT GT-3', Tq*vasa*-reverse: 5'-GAARGARACKGCYCKYCCDRTGTTKCCACA-3') that were designed using highly conserved regions of *vasa* homologues from *Drosophila*, zebrafish, medaka, rainbow trout, *Xenopus*, mouse and rat. The PCR reaction was carried out using 1 µl of cDNA products, 0.8 µl of dNTP, 1 µl of each primer 10 µM, 1

µl of ExTaq buffer (Takara Biomedicals), and 0.25 units of ExTaq enzyme (Takara Biomedicals) in a total volume of 10 µl. Amplification was performed with an initial denaturation step of 3 min at 94°C followed by 35 cycles as follows: denaturation for 30 s at 94°C, annealing for 30 s at 63.9 °C and extension for 1 min at 72°C. The final extension was performed for 3 minutes at 72 °C. PCR products were electrophoresed on 0.7% agarose gel and the DNA fragment that showed the predicted molecular weight was isolated using a Gelpure DNA Purification Kit (Gene Mate). The PCR-amplified DNA fragment was cloned into plasmid vector pGEM T-Easy (Promega) and sequenced.

2.3. Rapid Amplification of cDNA Ends (RACE)

To isolate a full-length cDNA, 3'-RACE and 5'-RACE system were performed (Invitrogen). After determining the DNA sequence of a partial *vasa* cDNA fragment, two specific primers for tambaqui *vasa* cDNA were synthesized (Tqvasa-3'UTR-Forward: 5'-CAACTACAAGCATCCATGGTGACCG GGAA-3', Tqvasa-5'UTR-Reverse: 5'-CCAAGTAACGAAGTTTACTTAGTCCGACCT-3') and used together with the primers 3' and 5' RACE provided by the kit. The PCR reaction was carried out using 1 µl of cDNA products, 0.8 µl of dNTP, 1 µl of each primer 10 µM (5' primer RACE + Tq*vasa*-5'UTR-Reverse; 3' primer RACE + Tq*vasa*-3'UTR-Forward), 1 µl of ExTaq buffer (Takara Biomedicals), and 0.25 units of ExTaq enzyme (Takara Biomedicals) in a total volume of 10 µl. Five reaction cycles were conducted, with each cycle consisting of 30 sec at 94 °C and 3 min at 68 °C. After, thirty five reaction cycles were conducted, and each cycle consisted of 30 sec at 94 °C, 30 sec at 65 °C, and 3 min at 72 °C, except that the first denaturation was carried out for 3 min at 94 °C and the last elongation reaction was carried out for 3 min at 72 °C. The PCR-product was diluted 100x and used as DNA sample for nested PCR. The PCR reaction was the same as the first PCR except that the annealing temperature was 62°C and the first five reaction cycles were not used. The provided 3'primer RACE nested and 5' primers RACE nested were used together with specific tambaqui *vasa* primer (Tq*vasa*-3'nested-Forward: 5'-CCGGGAACAACGAGAGCGAGAGAAAGCTCTT-3' and Tq*vasa*-5'nested-Reverse: 5'-GTCCGACCTTTCCACGACCAATGATGTCCA-3'). The dC-tailing reaction and PCR were performed according to the manufacturer's protocol.

Both the 3’ and 5’ RACE products were cloned into pGEM T-Easy vector (Promega) and used for DNA sequencing.

2.4. Histology and In Situ Hybridization (ISH)

Fragments of tambaqui testes and ovaries were fixed with Bouin’s solution, cut into 4 µm-thick sections using standard paraffin-embedding methods, and stained with hematoxylin and eosin. Localization of *tvasa* mRNA was analyzed by *in situ* hybridization on the adjacent sections, as previously described (Nagasawa et al., 2009). A 1292 base pair (bp) cDNA fragment (from nucleotide number 542 to 1704; GenBank no. MG839489) of *tvasa* was used to synthesize an antisense RNA probe with digoxigenin-labeling.

3. Results

3.1. Isolation of the tvasa and phylogenetic analysis

The full sequence of tambaqui *vasa* gene (added on GenBank no. MG839489) has an open reading frame of 2010 bp and the sequence alignment with other species indicates that the *vasa* gene kept high similarity among species (Table 1). The open reading frame encoded 669 amino acids and contained eight consensus sequences for the DEAD protein family (Linder et al., 1989) (Fig. 1). The phylogenetic analysis comparing the deduced protein sequence of *tvasa* with those of related proteins from other species revealed that the sequence obtained in this study belongs to the clade of Vasa (Fig. 2). Almost 35% of the amino acid between the N-terminus and amino acid position 169 were glycine residues. This region contained 8 repeats of each arginine-glycine and arginine-glycine-glycine (Fig. 3).

Table 1. The identities of *tvasa* to other *vasa* homologs.

Species	Identity	Species	Identity
Pygocentrus nattereri	87%	*Clarias gariepinus*	77%
Cyprinus carpio	86%	*Sinocyclocheilus rhinocerous*	76%
Astyanax mexicanus	85%	*Rhamdia quelen*	76%
Salmo salar	83%	*Carassius auratus*	75%
Oncorhynchus mykiss	83%	*Danio rerio*	72%
Thunnus orientalis	82%	*Mus musculus*	65%
Ictalurus punctatus	77%	*Drosophila melanogaster*	50%

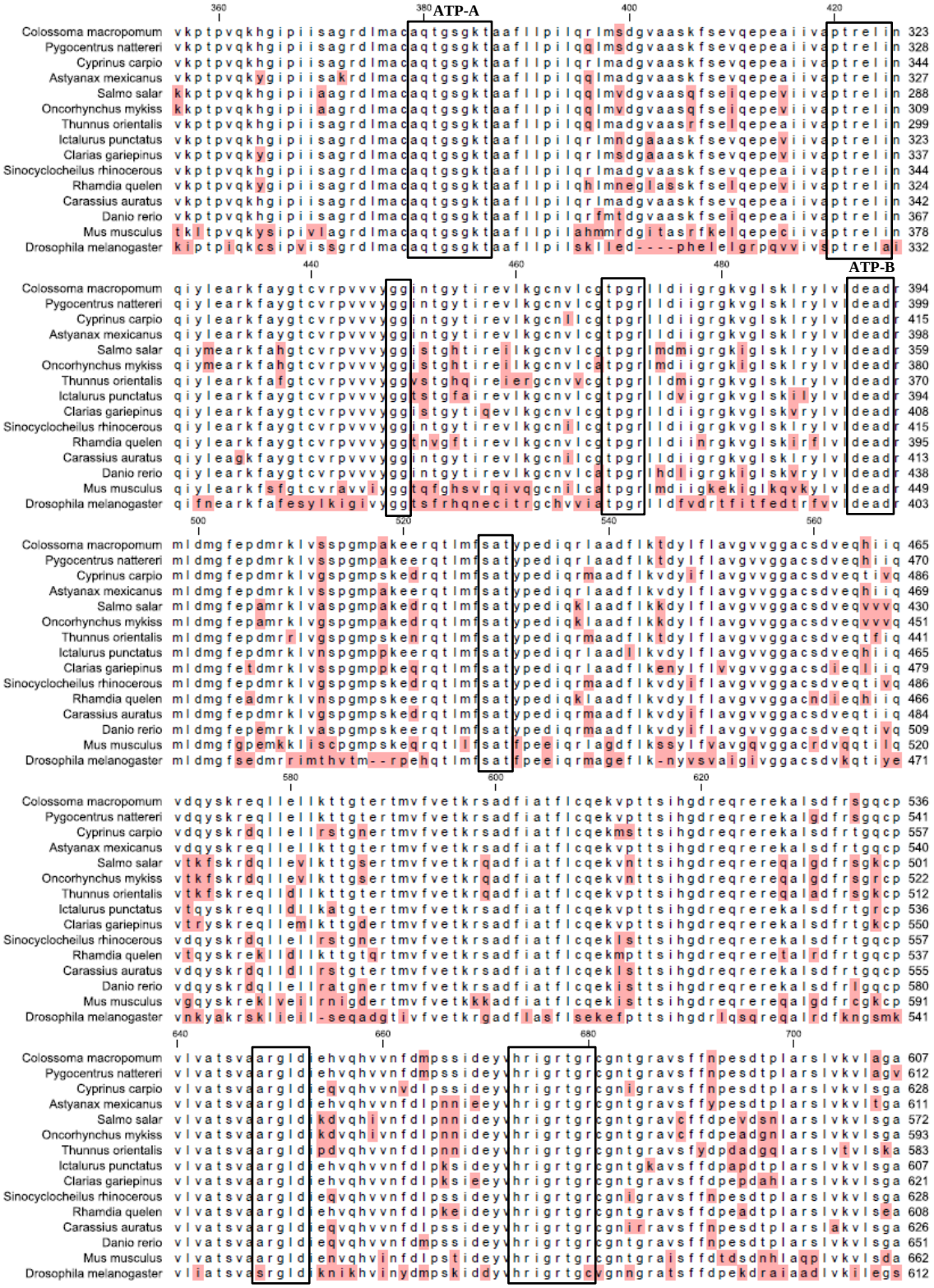

Fig. 1. Multialignment of the tVasa sequence with the other Vasa proteins. The different amino acid residues are highlighted in gray. Eight consensus sequences for the DEAD protein family, including and ATP-A motif and ATP-B motif, are boxed. The GenBank accession numbers of the aligned amino acid sequences were as follows: *Colossoma*

macropomum MG839489, *Pygocentrus nattereri* XP_017552779.1, *Astyanax mexicanus* XP_022536980.1, *Rhamdia quelen* AHL68988.1, *Ictalurus punctatus* XP_017345570.1, *Carassius auratus* AAX22126.1, *Sinocyclocheilus rhinocerous* XP_016377561.1, *Cyprinus carpio* AAL87139.2, *Danio rerio* XP_005156510.1, *Clarias gariepinus* ADK94762.1, *Salmo salar* XP_013998855.1, *Oncorhynchus mykiss* CDQ76412.1, *Thunnus orientalis* ABY77970.1, *Mus musculus* AAI44761.1, *Drosophila melanogaster* CAA31405.1.

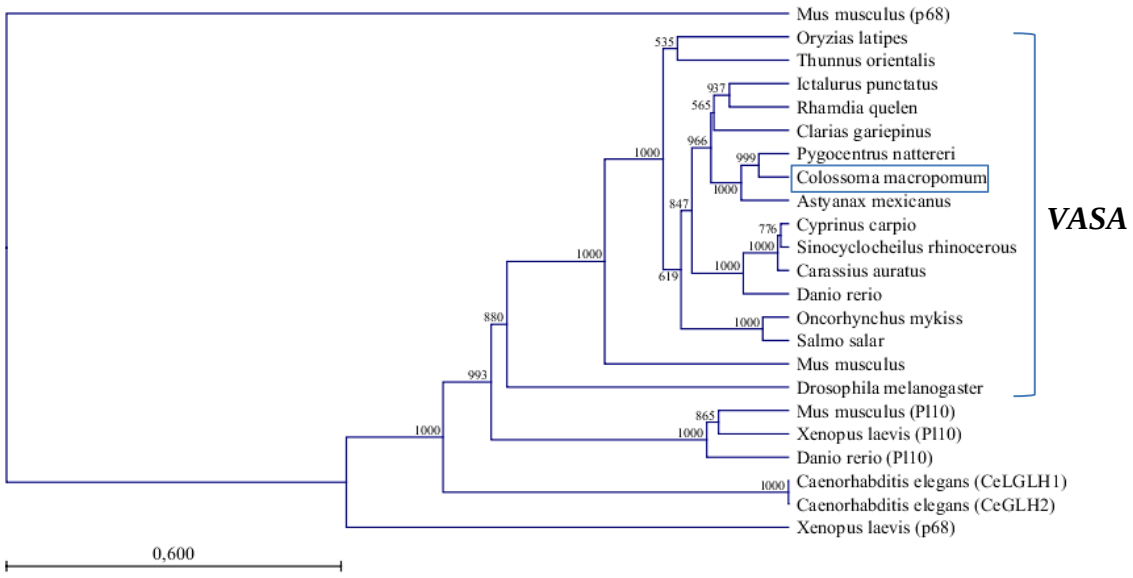

Fig. 2. Phylogenetic tree of DEAD-box proteins, using UPGMA method with CLC Main Workbench 6. Numbers next to nodes are bootstrap values based on 1000 replicates, which indicate the reliability of branches in each tree. The GenBank accession numbers of the aligned amino acid sequences were as follows: *Colossoma macropomum* MG839489, *Pygocentrus nattereri* XP_017552779.1, *Astyanax mexicanus* XP_022536980.1, *Rhamdia quelen* AHL68988.1, *Ictalurus punctatus* XP_017345570.1, *Carassius auratus* AAX22126.1, *Sinocyclocheilus rhinocerous* XP_016377561.1, *Cyprinus carpio* AAL87139.2, *Danio rerio* XP_005156510.1, *Clarias gariepinus* ADK94762.1, *Salmo salar* XP_013998855.1, *Oncorhynchus mykiss* CDQ76412.1, *Thunnus orientalis* ABY77970.1, *Mus musculus* AAI44761.1, *Drosophila melanogaster* CAA31405.1, *Xenopus laevis* NP_001080283.1 (Pl10), *Mus musculus* NP_149068.1 (Pl10), *Danio rerio* AAH59794.1 (Pl10), *Caenorhabditis*

elegans EAX88412 (CeGLH2), *Caenorhabditis elegans* AAC27384.1 (CeLGLH1), *Xenopus laevis* AAH82849.1 (p68), *Mus musculus* AAB53236.1 (p68).

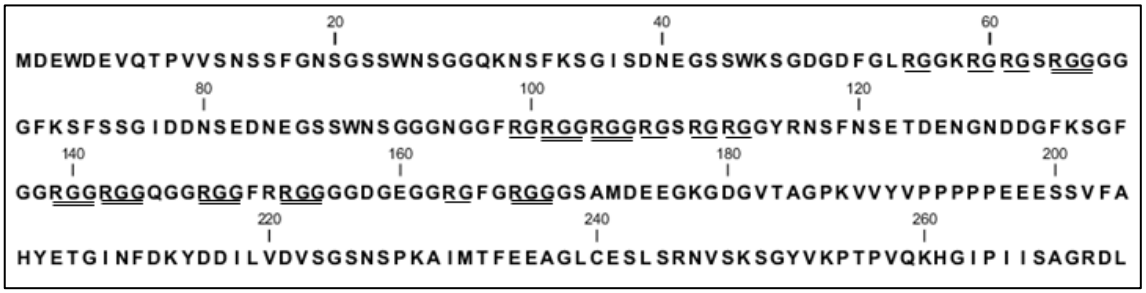

Fig. 3. N-terminal region of tVasa amino acid sequence. Arginine-glycine repeats and arginine-glycine-glycine repeats in the N-terminal region are underlined and double underlined, respectively.

3.2. Germ cells specific localization of tvasa mRNA

RT-PCR products showed that *tvasa* was detectable only in testes and ovaries of tambaqui (Fig. 4), and no expression was detected in other tissues. Further investigation of *tvasa* mRNA localization of tambaqui was conducted by ISH on serial ovary and testis sections and it showed strong positive signal in oocytes and spermatogonia (Fig. 5).

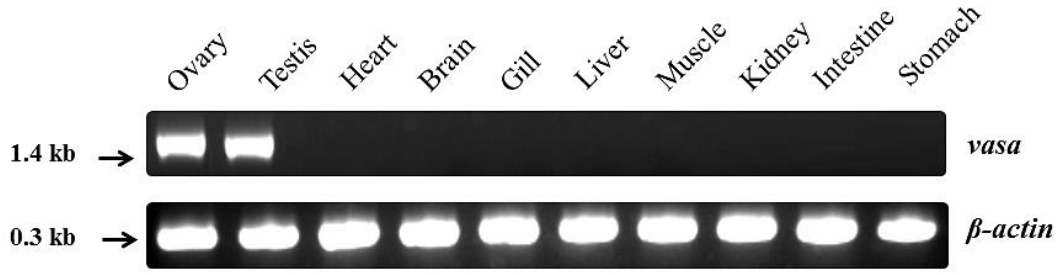

Fig. 4. Expression of *tvasa* in the tissues. β-actin served as a loading control

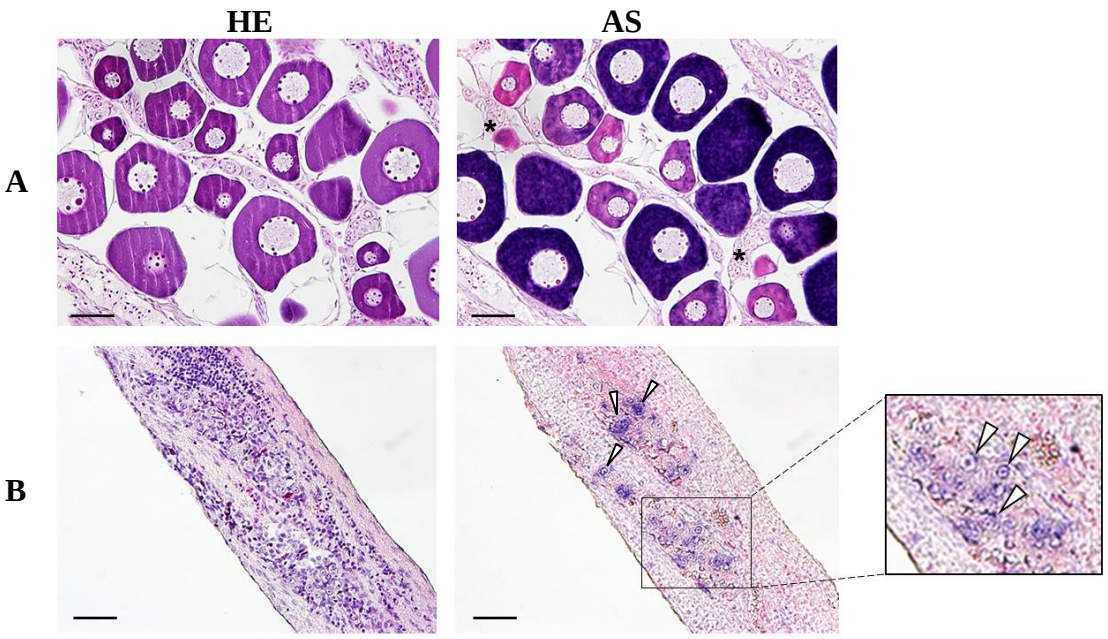

Fig. 5. *In situ* hybridization (ISH) analysis of *vasa* mRNA expression in gonad sections of *Colossoma macropomum.* (A) Ovarian expression of *vasa* gene showing strong signal in oocytes (with asterisks indicating the oogonia). (B) Testicular expression of *vasa* gene showing signal in spermatogonia (white arrows) and no signal in spermatocytes and spermatids. HE: hematoxylin-eosin staining; AS: antisense probe. Scale bar = 50µm.

4. Discussion

In tambaqui, there are presently no markers available for germ cells other than morphological characteristics to date. Since the *vasa* gene shows high specificity and it is widely conserved throughout the animal kingdom (Cao et al., 2012), the gene is the most documented molecular marker of germ cells in teleosts (Lin et al., 2012). Here we show that the amino acid sequence of the clone isolated in this study contained eight consensus sequences for the DEAD protein family, and the absence of mutation in the ATP-A motif (AXXXXGKT), the ATP-B motif (DEAD), the RNA unwinding motif (SAT) and the RNA binding motif (HRIGRXXR) (Pause & Sonenberg, 1992) suggesting it does not contain any contradiction as an helicase at least from its structural point of view since these motifs are required for ATP binding and hydrolysis (Tanner et al., 2003).

The tVasa had a glycine-rich region in the N-terminal, which was also found in *Drosophila* (Hay et al., 1988), mouse (Fujiwara et al., 1994), *Xenopus* (Komiya et al., 1994) and rainbow trout (Yoshizaki et al., 2000). It is believed that this region is characteristic of a single-stranded nucleic acid binding proteins such as RNA helicase (Hay et al., 1988). Moreover, the tVasa sequence contained acidic amino acid, aspartate (D) and glutamate (E), around the tryptophan (W) residues located at the N- and C-terminal regions, which is characteristic of Vasa protein (Blázquez et al., 2011).

The deduced amino acid sequence from the full-length cDNA showed 71% similarity and 50% identity with the *Drosophila melanogaster* Vasa, which was originally identified as a maternal-effect gene (Schupbach & Wieschaus, 1986), and 82% similarity and 65% identity with the *Mus musculus* Vasa, which has been functionally analyzed by gene knock-out techniques (Tanaka et al., 2000). Further, the phylogenetic analysis of the DEAD-box proteins family revealed that the tVasa belongs to a branch of vasa orthologues. Taken together with the results of the homology analyses and the consensus sequence analyses, this phylogenic tree analyses revealed that the cDNA clone isolated in this study is surely a *vasa* homologue of tambaqui.

The *vasa* expression in the tissues of tambaqui showed a restricted presence in the gonads, in agreement with the role of *vasa* as a translational regulator in germ line development (Braat et al., 1999). Similar germ cell-specific expression of *vasa* has been reported in zebrafish (Olsen et al., 1997; Yoon et al., 1997; Knaut et al., 2000), rainbow trout (Yoshizaki et al., 2000), tilapia (Kobayashi et al. 2000), medaka (Shinomiya et al., 2000), Gymnogobius (Saito et al., 2004), gibel carp (Xu et al., 2005), rare minnow (Cao et al., 2012), rice field eel (Ye et al., 2007), bluefin tuna (Nagasawa et al., 2009), grass carp (Li et al., 2010), European sea bass (Blázquez et al., 2011), turbot (Lin et al., 2012), Atlantic cod (Presslauer et al., 2012), blue tilapia (Xiao et al., 2013) and Lusitanian toadfish (Úbeda-Manzanaro et al., 2014).

In male of tambaqui, the *vasa* signal was present only in spermatogonia, and no signals were obtained in more advanced stage testes. Since some fish species also showed *vasa* expression in spermatocytes (Kobayashi et al., 2000; Xu et al., 2005; Ye et al., 2007; Cao et al., 2012; Lin et al., 2012; Xiao et al., 2013; Pacchiarini et al., 2013a; Pacchiarini et al., 2013b; Úbeda-Manzanaro et al., 2014; Yuan et al., 2014) and others showed its

predominant expression in spermatogonia (Yano et al., 2008), we need further studies to conclude *tvasa* expression in more developed germ cells.

In females of tambaqui, the absence of *vasa* mRNA in the oogonia could be due to its low level expression in this early stage of oogenesis and the increased expression in previtellogenic oocytes. This may be related to the fact that *vasa* mRNA works as maternally deposited factors (Pelegri et al., 1999), which play important roles for germ-line formation in the next generations (Schupbach & Wieschaus, 1986; Hay et al., 1990). Further, this expression profile in female germ-line suggests that the accumulation of *vasa* maternal factor could be occurred prior to the vitellogenesis starts.

The specific expression of *tvasa* in the germ cells makes the gene a useful marker for germ cell lineage and to study germ cell manipulations as well as basic studies to reveal the role of this protein during germ-line formation and early gametogenesis.

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Competing Interests

The authors declare no competing interests.

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CAPÍTULO III

First evidence of alternative splicing of *dead end gene in fish*

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First evidence of alternative splicing of *dead end* gene in fish

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Abstract

The Amazon species *Colossoma macropomum* (tambaqui) has been exploited commercially since the late 19th century due the appreciated flavor and rapid growth. However, declining of its natural resources due to overexploitation and the climate change is concerned. Although germ cell cryopreservation and transplantation can be a silver bullet to preserve their genetic resources, their germ cell behavior has not been analyzed to date. Here we isolated tambaqui *dnd* homolog (*tdnd*) and used it as a molecular marker for germ cells, in order to obtain basic information essential for transplantation. The amino acid sequence showed 98% similarity and 53% identity with the zebrafish *dead end*. Phylogenetic analysis and the presence of consensus motifs known for *dnd* revealed that the *tdnd* surely encodes *dnd* orthologue, and it was detectable only in testes and ovaries, showing strong positive signal in oocytes and undetectable signal in oogonia and meiotic cells of the testis. The sequencing analyses revealed three different transcripts of *tdnd* and the quantitative PCR showed that the expression profile of the full-length transcript had distinctive tendency to those of short transcripts, including the alternative splicing variant, suggesting that they play distinctive roles for germ-line development in this species.

Introduction

The control of eukaryotic gene expression is very complex and refined, and virtually all cells possessed by multicellular organisms have the same expression potential. During the cell differentiation processes, selective gene expression occurs and cells possessing identical genetic information acquire different physiological characteristics and functions. Although majority of gene expression controls are performed at transcription and translation levels, their fine tuning are also performed by alternative processing of messenger RNA (mRNA) precursors, generating different forms of mature mRNAs, depending on cell type[1,2].

The concept of alternative splicing was proposed for the first time in 1978[3] and this phenomenon explains the discrepancy between the number of genes encoding proteins and the number of the different proteins generated. Alternative splicing of precursor mRNA is a crucial mechanism to expand the complexity of gene expression, and it plays a substantial role in cellular differentiation and organism development. The constitutive splicing is a mechanism of RNA processing in which intragenic regions (introns) are removed and expressed regions (exons) are joined to form a mature RNA. Alternative splicing is a deviation from this preferred sequence where certain exons are skipped resulting in various forms of mature mRNA[4]. This important form of gene regulation contributes to the control of gene expression and increase the protein diversity[2,5,6]. In human genome, it is estimated that the proportion of genes that undergo alternative splicing reach to more than 95%[7-9].

Dead end gene (*dnd*), first identified in zebrafish[10], is a germ plasm component and encodes an RNA-binding protein crucial for the migration and survival of primordial germ cells (PGCs). In *dnd* depleted zebrafish embryos, the PGCs fail to acquire motility, lose specific marker gene expression and die within 24 hours post-fertilization[10] (Weidinger et al., 2003). *Dead end* has been shown to bind to the 3'-untranslated region (UTR) of mRNAs to displace micro-RNAs (miRNAs) that bind to adjacent sites on the same mRNA[11]. This protein is localized to perinuclear germ granules within PGCs, and several knockdown studies have confirmed the importance of *dnd* for the adequate development of the germ cells in teleost fish[10,12-16]. By *in situ* hybridization (ISH) with an antisense RNA probe, the *dnd* expression pattern in adults of medaka[17] and turbot[18] was found in germ cells at premeiotic and meiotic stages of

both sexes. In Pacific bluefin tuna, the localization of BFT*dnd* mRNA is restricted to type A spermatogonia, and not detected in other differentiated spermatogenic cells[19]. The use of the *dead end* gene as a germ cell marker has been increasingly recurrent in studies involving transplantation of germ cells. The production of chimeric teleosts through cell transplantation was developed in 1992[20] (Lin et al., 1992) and in 1994, this technology was used in mammals[21,22], becoming a valuable approach to the study of germ cell biology as well as research in the field of animal production, cell culture, reproductive medicine and production of transgenic animals[23-26]. The transplantation is also expected to be used as an alternative to preserve genetic resources of important fish species, such as *Colossoma macropomum*, the second largest finfish from the Amazon and Orinoco River basins in northern South America. Popularly known as tambaqui, this species is the second largest scaled fish in South America and it is among the most cultivated Neotropical fish species in fish farms[27]. Currently, only 1% of the animals in the natural environment has the size allowed for fishing (55 cm), showing that the species exploitation is above the maximum sustainable yield[28].

In order to develop a specific molecular marker for further identification of the germ cells, the tambaqui homolog of *dnd* was isolated, and expression patterns in tambaqui were examined in the gonads at different stages of development. In addition, we identified three transcripts of *dnd* in tambaqui and proposed that alternative splicing in this gene might interfere with protein function.

Results

Cloning of the *dead end* cDNA and phylogenetic analysis

The full length of *tdnd* (GenBank no. KY426013) has an open reading frame of 1194 bp that begins with the start codon ATG at the position 79, and the stop codon TAA at the position 1272. The amino acid sequence was inferred to encode 398 amino acid residues, and the sequence alignment with other species indicates that the *dead end* gene is highly conserved through the process of vertebrate evolution (Table 1; Fig.1). The predicted amino acid sequence has three RNA recognition motifs (RRM), RRM1 (AA 52–129), RRM2 (AA 131–211) and double-stranded RNA recognition motif (DSRM) (AA 314–394). RRM1 is the most conserved among the three domains, while DSRM is the least conserved domain. Phylogenetic analysis comparing the full-length cDNA of

tDnd with those of related proteins from other species revealed that tDnd sequence obtained in this study belongs to the clade of Dnd (Fig. 2).

Table 1. The identities of tDnd to other *dead end* proteins.

Species	**Identity**	**Species**	**Identity**
Pygocentrus nattereri	84%	*Danio rerio*	53%
Astyanax mexicanus	70%	*Oncorhynchus mykiss*	48%
Ictalurus punctatus	59%	*Salmo salar*	48%
Carassius auratus	57%	*Thunnus orientalis*	47%
Sinocyclocheilus grahami	56%	*Oryzias latipes*	46%
Cyprinus carpio	56%		

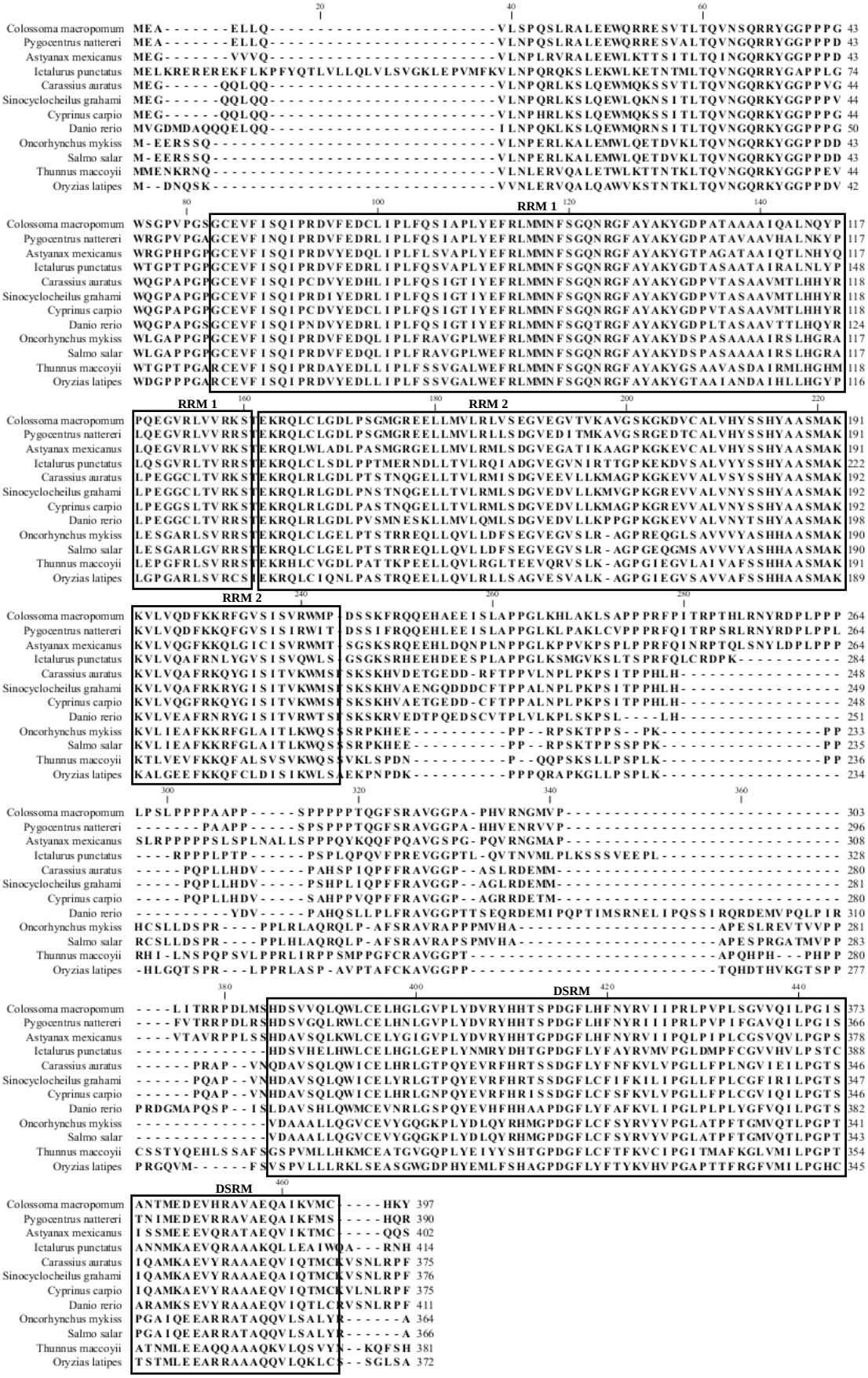
RRM 1
RRM 2
DSRM

Fig.1. Multialignment of selected *dnd* homologs. The RRM are shown in the frame, including RRM1, RRM2 and DSRM.

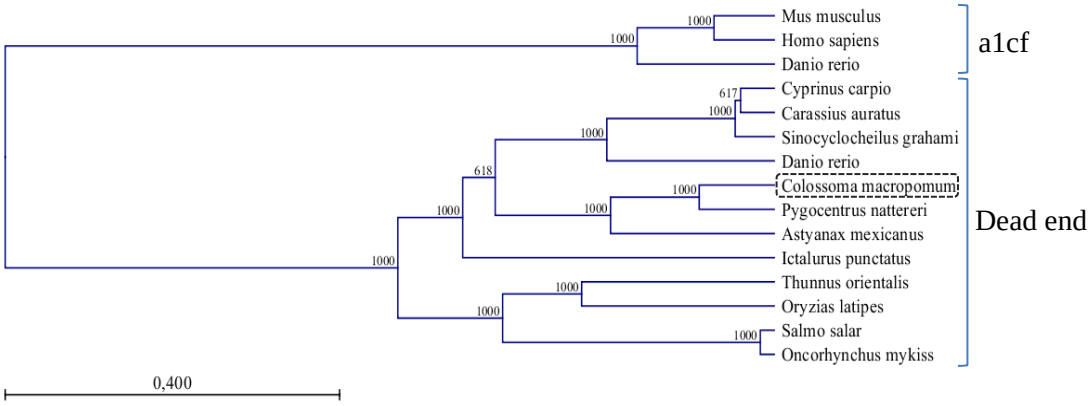

Fig. 2. UPGMA tree for the dead end and its related protein, a1cf family, based on dead end homologues of *C. carpio* (XP_018958879.1), *C. auratus* (AEX33122.1), *S. grahami* (XP_016098544.1), *D. rerio* (NP_997960.1), *C. macropomum* type 1 (AQY10189), type 2 (AST51843.1), type 3 (AST51844.1), *P. nattereri* (XP_017578011.1), *A. mexicanus* (XP_007253662.1), *I. punctatus* (XP_017340221.1), *T. orientalis* (KF128758.1), *O. latipes* (NP_001157988.1), *O. mykiss* (CDQ77433.1), *S. salar* (NP_001266060.1), and the apobec-1 factor of *H. sapiens* (NP_620311.1), *M. musculus* (NP_001074543.1) and *D. rerio* (XP_009304711.1).

Genomic DNA sequence of *tdnd*

The genomic DNA sequence of *tdnd* was analyzed to verify the spliced site of the transcript found in this study. After the genomic DNA sequence analyses and alignment with other species, we noticed that an exon of *tdnd* corresponding to exon 4 of all other known *dnd* homologues split into 2 exons and new intron was inserted (Fig. 3, exon 4' and 4''). Alignment of *dnd* locus of various vertebrates were shown in Fig. 3 and revealed that among these species, only tambaqui has segmentalized exon 4.

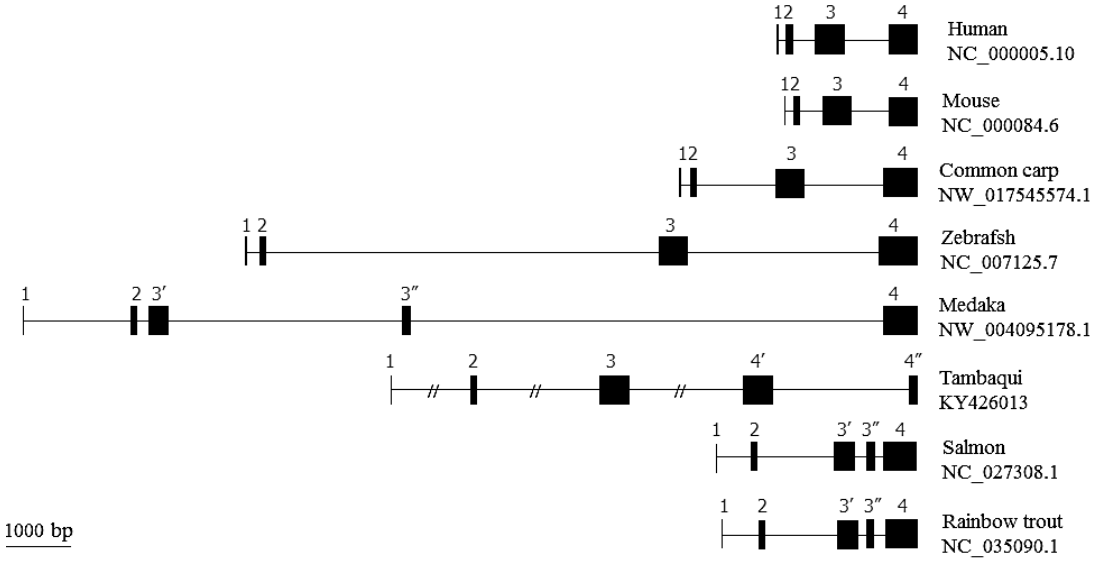

Fig. 3. Schematic drawing of exon-intron structure of various vertebrates. Predicted exons are shown in black box and introns in thin line.

Gel electrophoresis of RT-PCR products showed fragments of different lengths of *tdnd* (Fig.4a). Further, the sequencing of each fragment revealed that the *tdnd* gene has three transcripts, since smaller two fragments had nearly identical length and could not be distinguished on the electrophoretic pattern. The alignment of the obtained sequences showed that type 1 represents the complete sequence of the *tdnd* gene, while type 2 does not contain exon 3 (aa 43 to 196), and type 3 has a missing part of exon 4 and 5 (aa 209 to 366). The amino acid alignment of the three types of transcripts shows the limit of each exon (red arrow), and the missing sequences in the types 2 and 3 (Fig.4b).

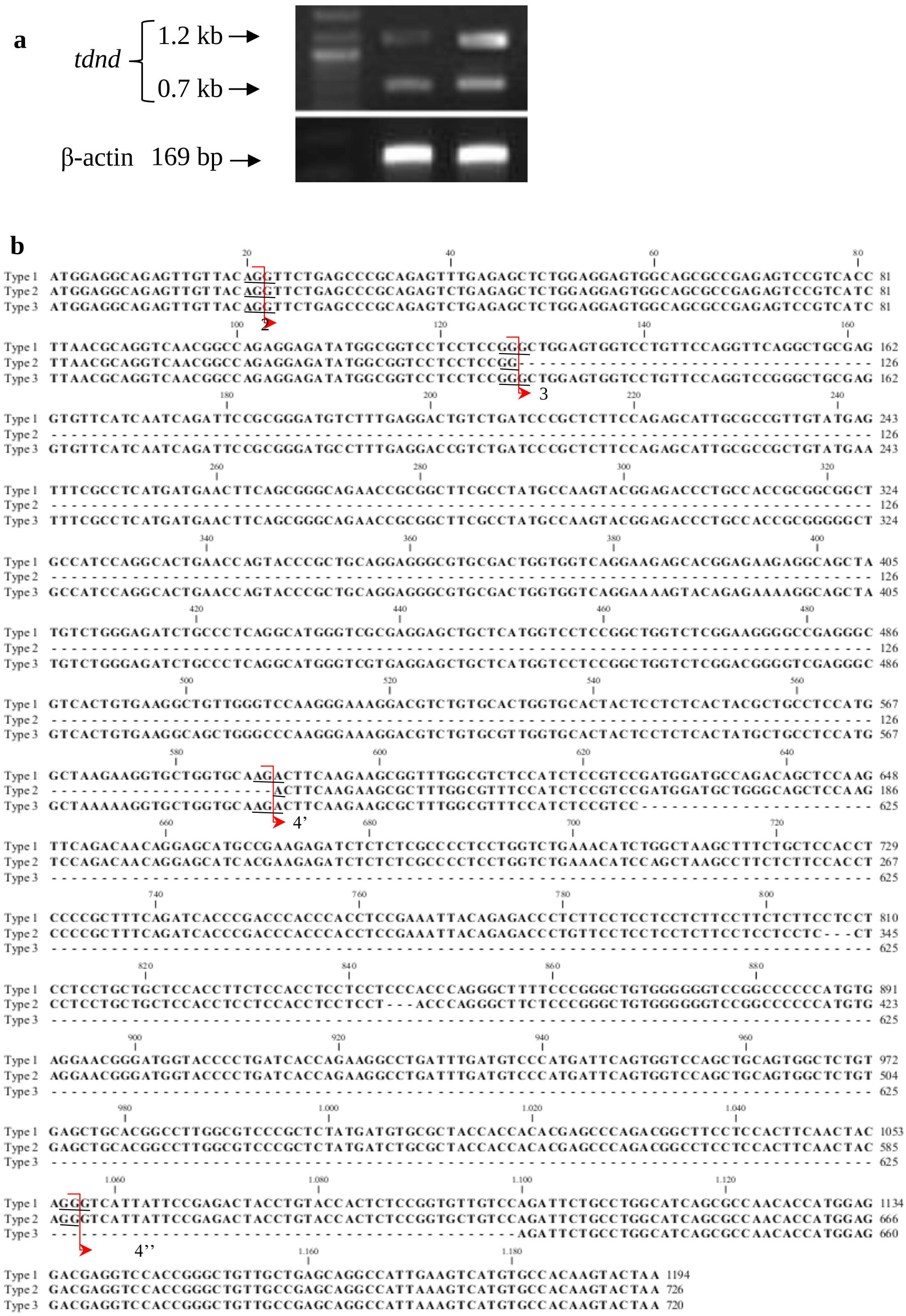

Fig. 4. Different transcripts of the *dead end* gene. (a) RT-PCR of *tdnd* using testicular and ovarian cDNA, showing different lengths of transcripts. (b) Nucleotide sequence

alignment of the three transcripts. The red arrows delimit each exon, with the donor and acceptor site underlined.

To determine tissue distribution profile of each transcript, primers specific to each transcript were designed to distinguish them (Fig. 5a). The results show that all transcripts were detectable only in testes and ovaries of tambaqui (Fig. 5b), and no expression was detected in other tissues including heart, brain, gill, liver, muscle, kidney, intestine and stomach.

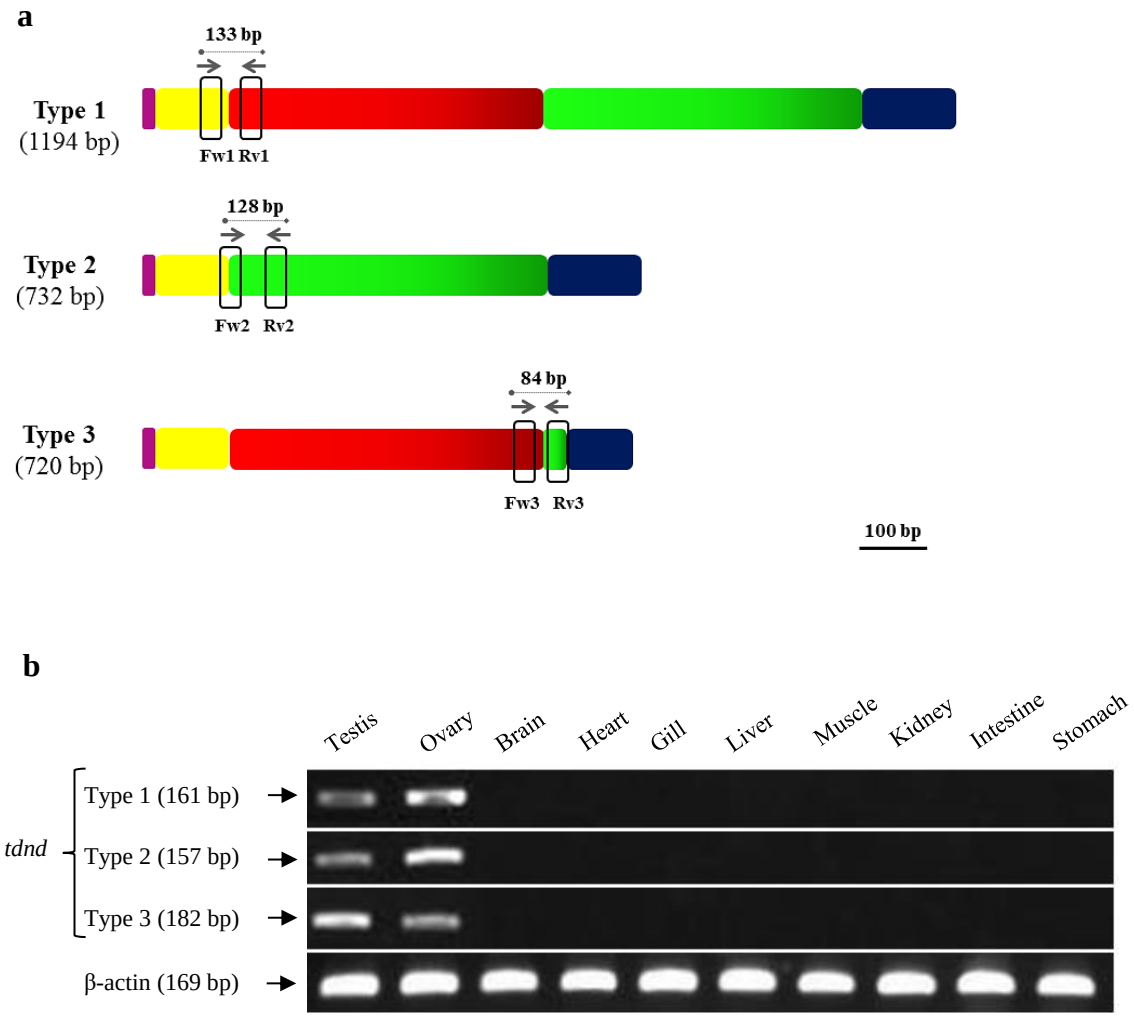

Fig. 5. (a) Schematic drawing of the position and length of each specific primer. The different colors represent each exon in the gene. (b) Expression of *dnd* transcripts in various tissues of tambaqui.

Quantitative PCR

The quantitative analysis with testes and ovaries of different aged animals showed that the three transcripts presented distinctive profiles of expression, although the relative quantification has shown a wave behavior (Fig. 6). Expression profile of the full-length transcript (Type 1) showed distinctive tendency to those of short transcripts (Type 2 and 3), including the alternative splicing variant (Type 2). On the other hand, those of Type 2 and Type 3 were quite similar, though their absolute values varied.

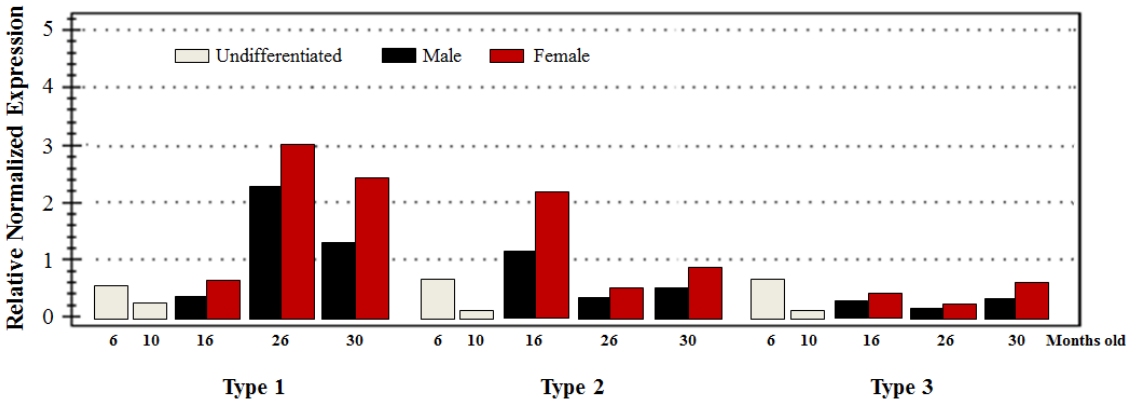

Fig. 6. Relative normalized expression of the *dead end* gene transcripts at different ages of tambaqui.

Specific expression of *tdnd*

Further investigation of *tdnd* mRNA localization of tambaqui was conducted by *in situ* hybridization with Type 1 *dnd* probes on serial sections of tambaqui gonads. In ovarian section, peri-nuclear stage oocytes showed clear positive signals. Further weak signals were detected also in immature spermatogonia (Fig. 7).

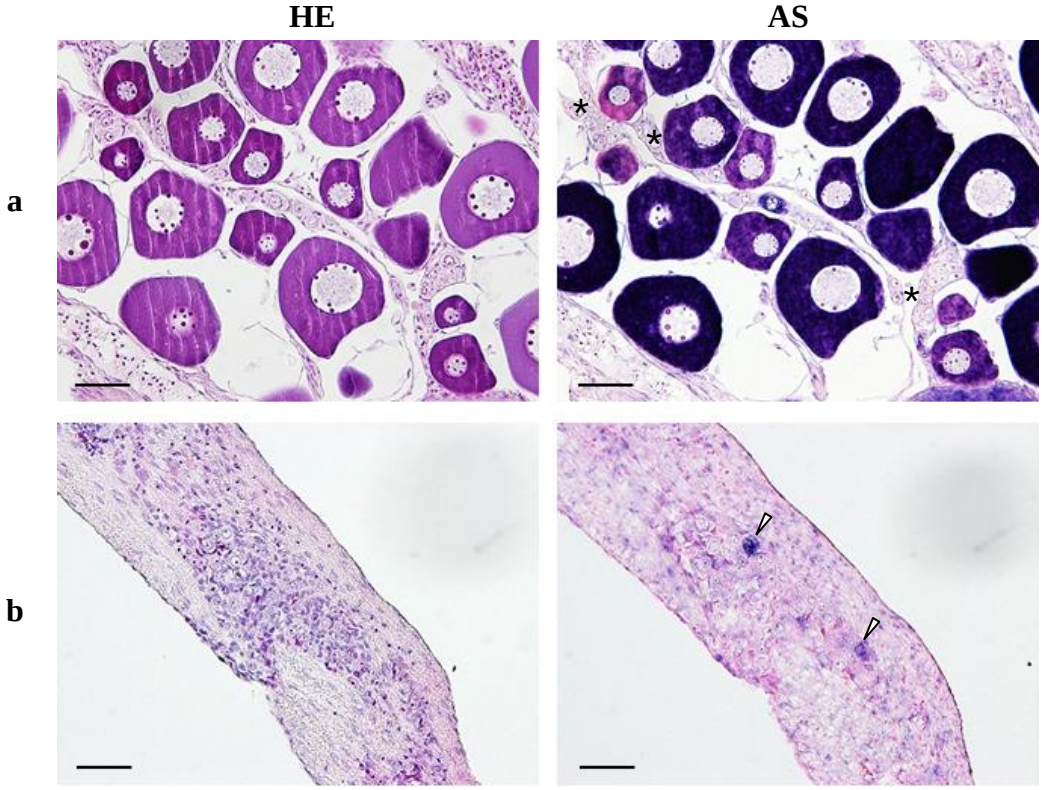

Fig. 7. *In situ* hybridization (ISH) analysis of *dead end* type 1 in gonad sections of *Colossoma macropomum.* (a) Strong expression of *tdnd* gene detected in pre-vitellogenic oocytes and no signal in oogonia (asterisk). (b) Testicular expression of *tdnd* gene showing weak signal in some spermatogonia (white arrow). HE: hematoxylin-eosin staining; AS: antisense probe. Scale bar = 50 µm.

Discussion

Here we identified and characterized the *dead end* cDNA as a candidate for germ cell marker of tambaqui. Additionally, we found three transcripts of the gene being expressed in the gonads of male and female. The amino acid sequence deduced from the full-length cDNA showed 98% similarity and 53% identity with the zebrafish dead end, which has been functionally analyzed by gene knock down techniques[10] and in vitro intermolecular interaction analyses[29]. The phylogenetic tree analysis of the dead end-related proteins revealed that the clone isolated in this study belongs to the branch of dead end homologues. Furthermore, the deduced amino acid sequence contained two single-strand RNA recognition motifs (RRM) and one double strand RNA binding domain (DSRM), which are typical characteristics of the dead end family. These three

conserved domains are found in the all known *dnd* proteins, including red piranha (GenBank no. XM_017722544.1), Mexican tetra (XM_007253600.3), goldfish (JN578697.1), Golden-line barbell (XM_016243058.1), common carp (XM_019103334.1), zebrafish (AY225448.1), rainbow trout (DQ974158.1), southern Bluefin tuna (KP171240.1), Atlantic salmon (JN712911.1) and medaka (NM_001164516.1), Xenopus (AAX84947.1) and mouse (NP_775559.2). Furthermore, *tdnd* were specifically and strongly expressed in oocytes, suggesting that *tdnd* is a germ-line gene and works as maternally inherited factor. Thus, the results from the sequence homology, phylogenic tree analyses, and domain structure, together with its specific expression in germ cell lineage fulfills the requirements of a *dnd* homolog, at least from a structural and expression point of view.

By sequence alignment of cDNA and gene of *tdnd*, we could reveal that *tdnd* is composed of 5 exons, instead of 4, which are typically observed in the most of the vertebrates, such as human (NC_000005.10), mouse (NC_000084.6) and zebrafish (NC_007125.7). By comparing *dnd* exon-intron structure of these species, we found that exon 4 of above-listed species was segmentalized into two exons, producing 5 exons collectively in tambaqui. The intron inserted between exon 4 and 5 of *tdnd* has typical splice donor site G-G-[cut]-G-U-R-A-G-U and splice acceptor site Y-rich-N-C-A-G-[cut]-G, in addition to the branch sequence (Y-U-R-A-C), which is located 20-50 nucleotides upstream of acceptor site (Genbank no. MG879029). When the exon-intron structure is analyzed in various teleosts, it is possible to observe that *dnd* of cyprinid fish have four exons, while other species, including *tdnd* present five exons. The position of additional introns suggests that the intron insertion process occur independently in *tdnd* from those of salmonids and medaka, since tambaqui has additional intron in the exon 4 and salmonids and medaka has it in the exon 3. At this moment, we cannot discuss about evolutional processes of these events since exon-intron structure information is still scarce in teleosts.

In this study, three types of transcripts were identified from *tdnd*. Even though the alternative splicing occurs with high frequency in the pre-mRNA composed of more than one exon, here, we report for the first time the occurrence of alternative splicing in the *dead end* gene, which generated at least one additional isoform with different expression profile. Although the recognition of each exon and intron in tambaqui

showed that type 2 was generated from the alternative splicing of the third exon, the truncated isoform 3 showed a different pattern of splicing compared to those documented so far. Although we could not figure out the mechanisms how the truncated isoform 3 was produced in this study, it will be important task in future studies.

It is known that alternative splicing is one of the main sources of proteomic diversity in multicellular eukaryotes, by which mRNAs with different functions can be synthesized from a single gene, although not all alternatively spliced transcripts produce functional proteins. In addition to being a mechanism for proteomic expansion, alternative splicing appears to modulate a function of the gene by adding or removing protein domains, affecting a protein activity or by altering a transcriptional stability or a resulting protein[30-32]. This process involves the differential use of splice sites to create distinct proteins and our results showed that isoforms 2 and 3 of the *dead end* gene were generated by different splice sites. The third exon of the type 2 isoform is completely replaced by an intron, being classified as "Exon skipping"[8], and this loss caused the complete exclusion of the RRM1 and the loss of 62 amino acids of RRM2, which represents almost 80% of its composition. In the type 3 transcript, 9% (7 amino acids) of RRM2 and 64% (51 amino acids) of the DSRM was excluded from mature mRNA during transcription. Although it has been reported that some RNA binding proteins may lose the RRM and keep the function through other well-conserved and structurally important residues[33], the loss of the RRM in the transcripts 2 and 3 may inactivate the protein, since the domains play a crucial role for the *dnd* function and could be involved in targeting the protein to its normal location in the cell[34].

The alternative splicing in *tdnd* may be related to the down regulation of the gene function[35-41], such as dominant-negative inhibition, since the absence of the above-mentioned motifs, that is important for their function, may impair the recognition of the molecule, causing the loss of the protective function of the *dead end* against the miRNA, which acts as post-transcriptional silencers. This function of protection is given by the fact that dead end interacts with the mRNA molecule by neutralizing the function of several miRNAs in the PGCs, by similarity, preventing them from associating with their target sites[11]. Furthermore, in most samples analyzed, we could observe that the relative expression of isoform 1 is inversely proportional to the relative expressions of the splicing variants 2 and 3. This behavior leads us to propose that the production of

the alternative forms of the *dead end* transcripts in tambaqui is regulated differently from the production mechanism of the full-length transcript (type 1).
In any case, specific expression of all three variants in gonads may suggest their importance in germ cell development. Further precise analyses will be needed to conclude the specific expression of each variant in the lineage of germ cells, since we could not detect each variant by using the RNA probe in *in situ* hybridization study.
In captivity, tambaqui reaches the sexual maturity after 3 years old but in the histology, it is possible to see oocytes and sperm around 12 months old. The increase in the expression of isoform 1 as the individual reaches sexual maturity was expected, since self-renewal and duplication of germ stem cells in seasonal reproduction species are more present before and after spawning or spermiation.
Although some animals are physiologically prepared to reproduce, the absence of abiotic factors may cause the regression of mature cells and this phenomenon can explain the low relative expression of the variant 1 at the age of 16 months (Vasconcelos et al., Unpublished results).
More comprehensive studies using wider range of target species are required to fully understand this phenomenon that is critically important for production of variety of proteins using limited size of their genomes. The maintenance of the three isoforms of the *tdnd* may suggest their biological significance in this species, although more precise studies will be needed to conclude.

Methods

RNA isolation

Fragments of ovary, testis, heart, brain, gill, liver, muscle, kidney, intestine and stomach were collected from 6, 10, 16, 26 and 30 months-old tambaqui, *Colossoma macropomum*. The animals were dissected after deep anesthetization followed by immediate decapitation. Total RNA was extracted from the ovaries and testis of tambaqui using a QuickPrep Total RNA Extraction Kit (Amersham Pharmacia Biotech). The quality of the isolated RNA was examined by spectrophotometer and denaturing gel electrophoresis.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Three micrograms of total RNA were used for cDNA synthesis. First strand cDNA was synthesized by means of Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech) with the adapter-oligo dT primer (5'-CTGATCTAGAGGTACCGG ATCC-oligo dT- 3'). RT-PCR was performed with degenerate primers (*dnd*-Fw1 and *dnd*-Rv1) that were designed using the highly conserved regions of *dnd* homologs from various fish species. The PCR reaction was carried out using 1 µl of cDNA products, 0.8 µl of dNTP, 1 µl of each primer (10 µM), 1 µl of 10x ExTaq buffer (Takara Biomedicals) and 0.25 units of ExTaq enzyme (Takara Biomedicals) in a total volume of 10 µl. Amplification was performed with an initial denaturation step of 5 min at 94°C followed by 35 cycles as follows: denaturation for 30 s at 94°C, annealing for 30 s at 63.9°C and extension for 1 min at 72°C. The final extension was performed for 3 minutes at 72°C. PCR products were electrophoresed on a 0.7% agarose gel and the DNA fragment that showed the predicted molecular weight was isolated using a Gelpure DNA Purification Kit (Gene Mate). Amplified cDNA fragments were cloned into the pGEM T-Easy Vector (Promega) and sequenced. All primers are shown below.

Table 1. Description of the primers used

Purpose	Gene	Primer name	Sequence
Cloning	*tdnd*	*tdnd*-deg-Fw-1	5'- GGSTGTGAGGTTTTCATCAGTCAG
		tdnd-deg-Rv-1	5'- CACAWGGTTTGGATCACCTGCTCA
5'RACE	*tdnd*	*tdnd*-5RACE-1	5'-GTCGAAGTTCATCATGAGGCGAAACTCATACAA
		tdnd-5RACE-2	5'- ACTCATACAACGGCGCAATGCTCTGGAAGA
3'RACE	*tdnd*	*tdnd*-3RACE-1	5'- GATGGTACCCCTGATCACCAGAAGGCCTGA
		tdnd-3RACE-2	5'- GAAGGCCTGATTTGATGTCCCATGATTCAGT
RT-PCR	*tdnd*	*tdnd*-total-Fw	5'- ATGGAGGCAGAGTTGTTACAGGTTCTGAG
		tdnd-total-Rv	5'- CTTGTGGCACATGACTTTAATGGCCTGCT
RT-PCR and qPCR	*actb*	actin-Fw	5'- CGTGATGGACTCTGGTGATG
		actin-Rv	5'- TCACGGACAATTTCCCTCTC
Alternative Splicing and qPCR	*tdnd*	Fw1	5'- CAGGTCAACAGCCAGAGGAG
		Rv1	5'- GGAAGAGCGGGATCAGACAG
		Fw2	5'- AGAGACCCTGTTCCTCCTCC
		Rv2	5'- ACCATCCCGTTCCTCACATG
		Fw3	5'- CCTCTCACTATGCTGCCTCC
		Rv3	5'- TCTGGACGGAGATGGAAACG
Genomic DNA	*tdnd*	Genomic-Fw	5'- CTTCAAGAAGCGGTTTGGCGTCTCCATCTCC
		Genomic-Rv	5'- TTGGCGCTGATGCCAGGCAGAATCTGGAC

Rapid Amplification of cDNA Ends (RACE)

To isolate a full-length cDNA, 5' and 3'rapid amplification of cDNA ends (RACE) was performed with a GeneRacer Kit (Life Technologies) according to manufacturer's instructions, using gene-specific primers for 5'RACE (*tdnd*-5'RACE-1 and *tdnd*-5'RACE-2) and 3'RACE (*tdnd*- 3'RACE-1 and *tdnd*-3'RACE-2). Amplified RACE products were cloned into the pGEM T-Easy Vector (Promega, Madison) and sequenced. The deduced amino acid sequences of *tdnd* and related molecules from various species obtained from GenBank were used in construction of phylogenetic tree, performed as described previously[41]. Touchdown and Nested PCR were used to reduce background amplification, and increase the specificity and sensibility of RACE products.

Histology and *In Situ* Hybridization

Fragments of tambaqui testes and ovaries were fixed with Bouin's solution, cut into 4 µm-thick sections using standard paraffin-embedding methods, and stained with hematoxylin and eosin. Localization of *tdnd* mRNA was analyzed by *in situ* hybridization on the sections, as previously described[42]. An 1194 base pair (bp) cDNA fragment of *tdnd* was used as a template to synthesize antisense RNA probe.

Genomic DNA

To study the alternative splicing of *tdnd* type 3, the genomic DNA was extracted using Gentra Puregene Tissue Kit (QIAGEN) as described in the manufacturer's protocol. Genomic DNA encoding *tdnd* were amplified by PCR using 1 µl of genomic DNA product, 0.8 µl of dNTP, 1 µl of each primer (10 µM), 1 µl of 10x ExTaq buffer (Takara Biomedicals) and 0.25 units of ExTaq enzyme (Takara Biomedicals) in a total volume of 10 µl. Amplification was performed with an initial denaturation step of 5 min at 94°C followed by 35 cycles as follows: denaturation for 30 s at 94°C, annealing for 30 s at 66°C and extension for 1 min at 72°C. The final extension was performed for 3 minutes at 72°C. PCR products were electrophoresed on a 0.7% agarose gel and the DNA fragment that showed the predicted molecular weight was isolated using a Gelpure DNA Purification Kit (Gene Mate). Amplified genomic DNA fragments were cloned

into the pGEM T-Easy Vector (Promega, Madison) and sequenced. The primers used are shown in Table 1.

Quantitative PCR

To compare the expression profiles of each isoform at different ages of tambaqui, qPCR was performed using the Ssoadvanced universal sybr green supermix kit (Bio-Rad) following the manufacturer's protocol. Quantifications of *tdnd* type 1, type 2 and type 3 were performed by ΔΔCt method with *actb* as an internal control. The qPCR reaction was carried out using 1 µl of cDNA products, 0.2 µl of each primer (10 µM), 5 µl of ssoadvanced universal SYBR green supermix (2x) and 3.6 µl of nuclease-free H_2O, in a total volume of 10 µl. Amplification was performed with an enzyme activation step for 3 min at 95°C followed by denaturation for 3 s at 95°C and annealing for 20 s at 60°C. The melting curve was added after 40 cycles of amplification.

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Author Contributions

A.C.N.V. designed and conducted all the experiments, analyzed the data and wrote the manuscript under the supervision of G.Y.; A.O. and M.M. helped in the execution and analysis of some experiments; D.P.S.J. designed the experiments; G.Y. designed and supervised all the experiments, and contributed with reagents/materials/analysis tools.

Competing Interests

The authors declare no competing interests.

4. Considerações finais

Uma das formas de preservação da espécie *Colossoma macropomum* é o transplante de células germinativas para outras espécies parentais ou a criopreservação das células germinativas indiferenciadas. Para atingir este objetivo, o estudo destas células é indicado para aperfeiçoar a técnica e garantir melhores resultados.

Com base nas análises de expressão gênica por PCR e hibridização *in situ*, o presente trabalho mostrou que os genes *vasa* e *dead end* podem ser usados como potenciais marcadores moleculares das células germinativas do tambaqui. Nas análises de hibridização *in situ*, ambos apresentaram um padrão de expressão similar, com expressiva presença nos oócitos pré-vitelogênicos e fraca detecção nas espermatogônias. A ausência de sinalização nas células indiferenciadas pode ser devido à baixa expressão genética nestes tipos celulares, entretanto, os genes estudados ainda podem ser considerados como potenciais marcadores moleculares em estudos envolvendo descrição e comportamento de células da linhagem germinativa.

Além de ser um estudo pioneiro no que diz respeito ao isolamento dos genes específicos das células germinativas nesta espécie, o presente trabalho relatou pela primeira vez, a ocorrência de *splicing* alternativo do gene *dead end*, uma proteína de ligação crucial para a migração e sobrevivência das células germinativas primordiais. A presença de mais de uma isoforma pode estar relacionada com a regulação do gene, visto que na maioria das análises, a alta presença do transcrito tipo 1 implicou na expressão reduzida de seus variantes.

As sequências de ambos os genes obtidas neste estudo permitem a identificação das células germinativas do tambaqui, bem como a análise da expressão genética. Desta forma, estes resultados contribuem para a base do estudo da técnica de transplante para a preservação da espécie , e sugere que a expressão genética do *dead end* envolve um complexo processo regulatório que dá origem a três isoformas proteicas.

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APÊNDICES

Apêndice 1: Normas utilizadas para a preparação do Capítulo II

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Articles should ideally be no more than 11 typeset pages in length. As a guide, the main text (not including Abstract, Methods, References and figure legends) should be no more than 4,500 words. The maximum Article title length is 20 words. The Abstract — which must be no more than 200 words long and contain no references — should serve both as a general introduction to the topic and as a brief, non-technical summary of the main results and their implications.

For the main body of the text, there are no explicit requirements for section organization. According to the authors' preference, the text may be organized as best suits the research. As a guideline and in the majority of cases, however, we recommend that you structure your manuscript as follows:

Introduction
Results (with subheadings)
Discussion (without subheadings)
Methods

A specific order for the main body of the text is not compulsory and, in some cases, it may be appropriate to combine sections. Figure legends are limited to 350 words. As a guideline references should be limited to 60 (this is not strictly enforced). Footnotes should not be used.

We suggest that Articles contain no more than 8 display items (figures and/or tables). In addition, a limited number of uncaptioned molecular structure graphics and numbered mathematical equations may be included if necessary. To enable typesetting of papers, the number of display items should be commensurate with the word length — we suggest that for Articles with less than 2,000 words, no more than 4 figures/tables should be included. Please note that schemes are not used and should be presented as figures.

Authors must provide a competing interests statement within the manuscript file.

Submissions should include a cover letter, a manuscript text file, individual figure files and optional supplementary information files. For first submissions (i.e. not revised manuscripts), authors may incorporate the manuscript text and figures into a single file up to 3 MB in size; the figures may be inserted in the text at the appropriate positions, or grouped at the end. Supplementary information should be combined and supplied as a single separate file, preferably in PDF format.

The following file types can be uploaded for Article text: txt, doc, docx, tex, (pdf [first submissions only]).

A submission template is available in the Overleaf template gallery to help you prepare a LaTeX manuscript within the ***Scientific Reports*** formatting criteria.

Scientific Reports is read by scientists from diverse backgrounds. In addition, many are not native English speakers. Authors should, therefore, give careful thought to how their findings may be communicated clearly. Although a shared basic knowledge of science may be assumed, please bear in mind that the language and concepts that are standard in one field may be unfamiliar to non-specialists. Thus, technical jargon should be avoided and clearly explained where its use is unavoidable.

Abbreviations, particularly those that are not standard, should also be kept to a minimum. Where unavoidable, abbreviations should be defined in the text or legends at their first occurrence, and abbreviations should be used thereafter. The background, rationale and main conclusions of the study should be clearly explained. Titles and abstracts in particular should be written in language that will be readily intelligible to any scientist. We strongly recommend that authors ask a colleague with different expertise to review the manuscript before submission, in order to identify concepts and terminology that may present difficulties to non-specialist readers.

The format requirements of ***Scientific Reports*** are described below.

Scientific Reports uses UK English spelling.

Format of manuscripts

In most cases we do not impose strict limits on word counts and page numbers, but we encourage authors to write concisely and suggest authors adhere to the guidelines below. For a definitive list of which limits are mandatory please visit the submission checklist page.

Articles should be no more than 11 typeset pages in length. As a guide, the main text (not including Abstract, Methods, References and figure legends) should be no more than 4,500 words. The maximum title length is 20 words. The Abstract (without heading) - which must be no more than 200 words long and contain no references - should serve both as a general introduction to the topic and as a brief, non-technical summary of the main results and their implications.

The manuscript text file should include the following parts, in order: a title page with author affiliations and contact information (the corresponding author should be identified with an asterisk). The main text of an Article can be organised in different ways and according to the authors' preferences, it may be appropriate to combine sections.

As a guideline, we recommend that sections include an Introduction of referenced text that expands on the background of the work. Some overlap with the Abstract is acceptable. This may then be followed by sections headed Results (with subheadings), Discussion (without subheadings) and Methods.

The main body of text must be followed by References, Acknowledgements (optional), Author Contributions (names must be given as initials), Additional Information (including a Competing Interests Statement),

Figure Legends (these are limited to 350 words per figure) and Tables (maximum size of one page). Footnotes are not used.

For first submissions (i.e. not revised manuscripts), authors may choose to incorporate the manuscript text and figures into a single file up to 3 MB in size in either a Microsoft Word, LaTeX, or PDF format - the figures may be inserted within the text at the appropriate positions, or grouped at the end.

For revised manuscripts authors should provide all textual content in a single file, prepared using either Microsoft Word or LaTeX. We do not accept PDF files for article text for revised manuscripts. Figures should be provided as individual files.

Supplementary Information should be combined and supplied as a separate file, preferably in PDF format. The first page of the Supplementary Information file should include the title of the manuscript and the author list.

Authors who do not incorporate the manuscript text and figures into a single file should adhere to the following: all textual content should be provided in a single file, prepared using either Microsoft Word or LaTeX; figures should be provided as individual files.

The manuscript file should be formatted as single-column text without justification. Pages should be numbered using an Arabic numeral in the footer of each page. Standard fonts are recommended and the 'symbols' font should be used for representing Greek characters.

Methods

Where appropriate, we recommend that authors limit their Methods section to 1,500 words. Authors must ensure that their Methods section includes adequate experimental and characterization data necessary for others in the field to reproduce their work. Descriptions of standard protocols and experimental procedures should be given. Commercial suppliers of reagents or instrumentation should be identified only when the source is critical to the outcome of the experiments. Sources for kits should be identified. Experimental protocols that describe the synthesis of new compounds should be included. The systematic name of the compound and its bold Arabic numeral are used as the heading for the experimental protocol. Thereafter, the compound is represented by its assigned bold numeral. Authors should describe the experimental protocol in detail, referring to amounts of reagents in parentheses, when possible (eg 1.03 g, 0.100 mmol). Standard abbreviations for reagents and solvents are encouraged. Safety hazards posed by reagents or protocols should be identified clearly. Isolated mass and percent yields should be reported at the end of each protocol.

References

References will not be copy edited by ***Scientific Reports***. References will be linked electronically to external databases where possible, making correct formatting of the references essential.

References should be numbered sequentially, first throughout the text, then in tables, followed by figures; that is, references that only appear in tables or figures should be last in the reference list. Only one publication is given for each number. Only papers or datasets that have been published or

accepted by a named publication, recognized preprint server or data repository should be in the numbered list; preprints of accepted papers in the reference list should be submitted with the manuscript. Published conference abstracts and numbered patents may be included in the reference list. Grant details and acknowledgements are not permitted as numbered references. Footnotes are not used.

Scientific Reports uses standard ***Nature*** referencing style. All authors should be included in reference lists unless there are six or more, in which case only the first author should be given, followed by 'et al.'. Authors should be listed last name first, followed by a comma and initials (followed by full stops) of given names. Article and dataset titles should be in Roman text, only the first word of the title should have an initial capital and the title should be written exactly as it appears in the work cited, ending with a full stop. Book titles should be given in italics and all words in the title should have initial capitals. Journal and data repository names are italicized and abbreviated (with full stops) according to common usage. Volume numbers and the subsequent comma appear in bold. The full page range should be given (or article number), where appropriate.

Published papers:

Printed journals

Schott, D. H., Collins, R. N. & Bretscher, A. Secretory vesicle transport velocity in living cells depends on the myosin V lever arm length. ***J. Cell Biol***. **156**, 35-39 (2002).

Online only

Bellin, D. L. ***et al***. Electrochemical camera chip for simultaneous imaging of multiple metabolites in biofilms***.*** ***Nat. Commun***. **7**, 10535; 10.1038/ncomms10535 (2016).

For papers with more than five authors include only the first author's name followed by '***et al.***'.

Books:

Smith, J. Syntax of referencing in ***How to reference books*** (ed. Smith, S.) 180-181 (Macmillan, 2013).

Online material:

Babichev, S. A., Ries, J. & Lvovsky, A. I. Quantum scissors: teleportation of single-mode optical states by means of a nonlocal single photon. Preprint at https://arxiv.org/abs/quant-ph/0208066 (2002).

Manaster, J. Sloth squeak. ***Scientific American Blog Network*** http://blogs.scientificamerican.com/psi-vid/2014/04/09/sloth-squeak (2014).

Hao, Z., AghaKouchak, A., Nakhjiri, N. & Farahmand, A. Global integrated drought monitoring and prediction system (GIDMaPS) data sets. ***figshare*** https://doi.org/10.6084/m9.figshare.853801 (2014).

Acknowledgements

Acknowledgements should be brief, and should not include thanks to anonymous referees and editors, or effusive comments. Grant or contribution numbers may be acknowledged. Assistance from medical writers, proof-readers and editors should also be acknowledged here.

Author contributions

Scientific Reports requires an Author Contribution Statement as described in the Author responsibilities section of our Editorial and Publishing Policies.

Competing interests

A competing interests statement is required for all accepted papers published in ***Scientific Reports***. If there is no conflict of interest, a statement declaring this will still be included in the paper.

Data availability

Scientific Reports requires a Data Availability Statement to be included in the Methods section of submitted manuscripts (see 'Availability of materials and data' section for more information).

Figure legends

Figure legends begin with a brief title sentence for the whole figure and continue with a short description of what is shown in each panel in sequence and the symbols used; methodological details should be minimised as much as possible. Each legend must total no more than 350 words. Text for figure legends should be provided in numerical order after the references.

Tables

Please submit tables in your main article document in an editable format (Word or TeX/LaTeX, as appropriate), and not as images. Tables that include statistical analysis of data should describe their standards of error analysis and ranges in a table legend.

General figure guidelines

Authors are responsible for obtaining permission to publish any figures or illustrations that are protected by copyright, including figures published elsewhere and pictures taken by professional photographers. The journal cannot publish images downloaded from the internet without appropriate permission.

Figures should be numbered separately with Arabic numerals in the order of occurrence in the text of the manuscript. When appropriate, figures should include error bars. A description of the statistical treatment of error analysis should be included in the figure legend. Please note that schemes are not used; sequences of chemical reactions or experimental procedures should be submitted as figures, with appropriate captions. A limited number of uncaptioned graphics depicting chemical structures - each labelled with their name, by a defined abbreviation, or by the bold Arabic numeral - may be included in a manuscript.

Figure lettering should be in a clear, sans-serif typeface (for example, Helvetica); the same typeface in the same font size should be used for all figures in a paper. Use 'symbols' font for Greek letters. All display items should be on a white background, and should avoid excessive boxing, unnecessary

colour, spurious decorative effects (such as three-dimensional 'skyscraper' histograms) and highly pixelated computer drawings. The vertical axis of histograms should not be truncated to exaggerate small differences. Labelling must be of sufficient size and contrast to be readable, even after appropriate reduction. The thinnest lines in the final figure should be no smaller than one point wide. Authors will see a proof that will include figures.

Figures divided into parts should be labelled with a lower-case bold a, b, and so on, in the same type size as used elsewhere in the figure. Lettering in figures should be in lower-case type, with only the first letter of each label capitalized. Units should have a single space between the number and the unit, and follow SI nomenclature (for example, ms rather than msec) or the nomenclature common to a particular field. Thousands should be separated by commas (1,000). Unusual units or abbreviations should be spelled out in full or defined in the legend. Scale bars should be used rather than magnification factors, with the length of the bar defined on the bar itself rather than in the legend. In legends, please use visual cues rather than verbal explanations such as "open red triangles".

Unnecessary figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Figures should not contain more than one panel unless the parts are logically connected; each panel of a multipart figure should be sized so that the whole figure can be reduced by the same amount and reproduced at the smallest size at which essential details are visible.

Figures for peer review

At the initial submission stage authors may choose to upload separate figure files or to incorporate figures into the main article file, ensuring that any inserted figures are of sufficient quality to be clearly legible.

When submitting a revised manuscript all figures must be uploaded as separate figure files ensuring that the image quality and formatting conforms to the specifications below.

Figures for publication

Each complete figure must be supplied as a separate file upload. Multi-part/panel figures must be prepared and arranged as a single image file (including all sub-parts; a, b, c, etc.). Please do not upload each panel individually.

Please read the digital images integrity and standards section of our Editorial and Publishing Policies. When possible, we prefer to use original digital figures to ensure the highest-quality reproduction in the journal. For optimal results, prepare figures to fit A4 page-width. When creating and submitting digital files, please follow the guidelines below. Failure to do so, or to adhere to the following guidelines, can significantly delay publication of your work.

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Chemical and biological nomenclature and abbreviations

Molecular structures are identified by bold, Arabic numerals assigned in order of presentation in the text. Once identified in the main text or a figure, compounds may be referred to by their name, by a defined abbreviation, or by the bold Arabic numeral (as long as the compound is referred to consistently as one of these three).

When possible, authors should refer to chemical compounds and biomolecules using systematic nomenclature, preferably using IUPAC. Standard chemical and biological abbreviations should be used. Unconventional or specialist abbreviations should be defined at their first occurrence in the text.

Gene nomenclature

Authors should use approved nomenclature for gene symbols, and use symbols rather than italicized full names (for example Ttn, not titin). Please consult the appropriate nomenclature databases for correct gene names and symbols. A useful resource is LocusLink.

Approved human gene symbols are provided by HUGO Gene Nomenclature Committee (HGNC), e-mail: hgnc@genenames.org; see also www.genenames.org. Approved mouse symbols are provided by The Jackson Laboratory, e-mail: nomen@informatics.jax.org; see also www.informatics.jax.org/mgihome/nomen.

For proposed gene names that are not already approved, please submit the gene symbols to the appropriate nomenclature committees as soon as possible, as these must be deposited and approved before publication of an article.

Avoid listing multiple names of genes (or proteins) separated by a slash, as in 'Oct4/Pou5f1', as this is ambiguous (it could mean a ratio, a complex, alternative names or different subunits). Use one name throughout and include the other at first mention: 'Oct4 (also known as Pou5f1)'.

6. Vita

Ana Carina Nogueira Vasconcelos nasceu dia 08 de junho de 1987 no município de Divinópolis – MG, filha de Antonio Lucio Nogueira e Rosana Joyce de Vasconcelos Aguiar Nogueira.

Cursou o Ensino Fundamental e Médio no Colégio Anglo, no município de Divinópolis – MG. Em 2007, ingressou no Curso de Ciências Biológicas na Universidade Federal de Lavras (UFLA) e realizou experimentos na Universidad de Murcia, na Espanha. Em 2012 e 2013, cursou o Mestrado em Ciências Veterinárias na UFLA, período no qual realizou cursos de Biologia Molecular na Universidad Nacional de Rosário, na Argentina. Em 2014, iniciou o curso de Doutorado junto ao Programa de Pós-Graduação em Zootecnia da Universidade Federal do Rio Grande do Sul (UFRGS) na área de concentração Produção Animal, como bolsista CAPES.

No final do primeiro ano de doutorado, recebeu treinamento para executar a técnica de transplante de células-tronco germinativas em peixes, na Tokyo University of Marine Science and Technology (TUMSAT – Japão). No início de 2016, foi contemplada com uma bolsa de doutorado sanduíche pelo CNPq para realizar o seu experimento de doutorado na TUMSAT, onde aprendeu técnicas de Biologia Molecular, incluindo Engenharia Genética.