UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR

Rayan Silva de Paula

Utilização de ferramentas moleculares para detecção e controle de agentes bioincrustantes em hidrelétricas

BELO HORIZONTE
2022

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Tese de Doutorado apresentado ao Programa de Pós-Graduação em Biologia Celular do Departamento de Morfologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como requisito para obtenção do título de Doutor em Biologia Celular.

Orientadora: Professora Doutora Erika Cristina Jorge
Coorientadora: Doutora Mariana de Paula Reis Guimarães

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ATA DA DEFESA DE TESE DE DOUTORADO DE RAYAN SILVA DE PAULA

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Às **nove horas** do dia **29 de agosto de 2022**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"UTILIZAÇÃO DE FERRAMENTAS MOLECULARES PARA DETECÇÃO E CONTROLE DE AGENTES BIOINCRUSTANTES EM HIDRELÉTRICAS"**, requisito final para obtenção do grau de Doutor em Biologia Celular. Abrindo a sessão, a Presidente da Comissão, **Dra. Erika Cristina Jorge**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	**Instituição**	**Indicação**
Dra. Erika Cristina Jorge	UFMG	Aprovado
Dr. Mariana de Paula Reis Guimarães	Centro de Bioengenharia-Espécies Invasoras de Hidrelétricas (CBEIH)	Aprovado
Dra. Samyra Maria dos Santos Nassif Lacerda	UFMG	Aprovado
Dra. Lucília Souza Miranda	UFMG	Aprovado
Dra. Amanda Ferreira e Cunha	UFV	Aprovado
Dr. Fabiano Alcísio e Silva	CBEIH	Aprovado

Pelas indicações, o candidato foi considerado: Aprovado

O resultado final foi comunicado publicamente ao candidato pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 29 de agosto de 2022.**

Drª. Erika Cristina Jorge (Orientadora)

Dr. Mariana de Paula Reis Guimarães (Coorientadora)

Drª. Samyra Maria dos Santos Nassif Lacerda

Drª. Lucília Souza Miranda

Drª. Amanda Ferreira e Cunha

Dr. Fabiano Alcísio e Silva

Obs: Este documento não terá validade sem a assinatura do Coordenador

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AGRADECIMENTOS

À Profa. Erika Jorge por nossa jornada. Quem diria que conversas na janela, estando eu no 1º andar do Museu de Morfologia, e ela, no 2º andar, onde fica o laboratório, renderiam em mais de 10 anos de parceria científica! A conexão foi basicamente imediata e, acredito eu, mediada pelo fascínio e devoção à Embriologia. Obrigado por todo ensinamento, confiança, paciência e credibilidade em meu trabalho. Ingressei no grupo de pesquisa liderado pela Profa. Erika totalmente inexperiente, não sabia pipetar, quem dirá biologia molecular ou "fazer ciência".Em todos esses anos, a Profa. Erika nunca hesitou em compartilhar seu conhecimento com todosa sua volta, incentivando a cada um tornar-se sempre sua melhor versão. Nunca vou me esquecerda frase: "O conhecimento gera liberdade de escolha", proferida a uma de suas turmas do curso de Ciências Biológicas, e que acompanhei durante a disciplina de Estágio Didático. Essa frase evidencia o valor que a Profa. Erika atribui à educação e mostra sua excelência não apenas comoPesquisadora, mas, sobretudo, como Professora. Onde quer que eu esteja e/ou com o que ou quemeu estiver trabalhando, vou levar comigo toda essa bagagem e amadurecimento que adquiri durante esses anos de convivência. Serei eternamente grato a ela pelo espaço e confiança cedidosa mim, para que eu pudesse estabelecer e desenvolver o cientista e profissional que sou hoje. Obrigado por ser uma referência científica e pessoal que admiro e respeito imensamente.

À Mariana Reis Guimarães que conheci em 2015 e tenho o privilégio de trabalhar com maior proximidade desde 2018. Desde nossas primeiras trocas de e-mail e mensagens, salvei seucontato, em minha agenda, como Mariana Lama (que digamos ela não ter gostado muito quandodescobriu), uma vez que eu pensava que seríamos apenas colaboradores de um trabalho. No entanto, hoje, ela se tornou a Marivilhosa (sim, apelido de minha autoria), que mostra o carinho,admiração, devoção e orgulho em tê-la não apenas como colaboradora, coorientadora científica,mas como uma das melhores amigas que possuo. A Mari é um ser que emana amor, afeto e carinho para todos a sua volta, aquecendo o *heart* de qualquer pessoa. Eu tenho certeza de que eu ganhei uma amizade pelo restante da vida! Ainda vamos fazer muitos *tours* gastronômicos, idas à costureira para ajuste de peças estilosas, e, até mesmo, beber uma taça de vinho. Eu amo do fundo da minha *life* sua companhia e sua energia! Obrigado por ser uma inspiração profissional, científica e, sobretudo, humana.

Ao Prof. Antônio Valadão por ter acreditado em meu trabalho desde minha entrevista para ingressar à equipe do Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas (CBEIH) em 2014. Sua percepção acerca do "fazer ciência" e da valorização do cientista são fonte de inspiração. Aprendi com ele que colaborações bem estabelecidaas são indispensáveis e

que as mais diversas ideias devem ser ouvidas, respeitadas, ponderadas e aproveitadas a fim de dar seguimento à realização de um bom trabalho. Agradeço pela credibilidade e confiança em minhas propostas e pela liberdade concedida durante a vigência do projeto para que eu seguisseuma linha de pesquisa dentro do CBEIH. Todo pesquisador em formação deveria ter a oportunidade de debulhar-se sobre suas ideias e desbravar suas descobertas científicas a partir dessa perspectiva. Obrigado por enxergar e despertar em mim o potencial em ser um pesquisadore proporcionar experiências que me fizessem amadurecer cientificamente. Exemplo de liderançae coordenação científica a serem seguidas!

À Profa. Gerluza Silva que demonstra humanidade em um ambiente acadêmico que, infelizmente, mostra-se, muitas das vezes, governado por egos inflados e hostilidade. A Profa. Gerluza demonstra que realizar um trabalho com seriedade, respeito e dedicação não está dissociado em realizá-lo com alegria, bom humor e felicidade. Nossos bastidores, como ela costuma dizer, são recheados de festança e diversão, tornando tudo mais leve e agradável. Agradeço por ter sido, mesmo de forma não oficial, mas sempre de coração aberto, minha "orientadora" de extensão, me vinculando a um projeto que admiro incondicionalmente. Obrigado por se doar tanto e se propor a executar tudo com maestria, tanto no campo profissionalquanto pessoal. Seu carinho e consideração pelas pessoas é admirável!

Preciso agradecer, de alguma forma, ao Laboratório de Biologia Oral e do Desenvolvimento (LABODE) quanto espaço físico (racionalmente, é possível sentir isso?). Vi tantas configurações do laboratório ao longo desses 10 anos, participei das (longas) discussões para criação/aprovação da sigla e da logomarca (que eu acho um charme a parte). Obrigado por ser meu refúgio em tantos anos frequentando o ICB-UFMG. Um lugar com a energia tão receptiva é reflexo das pessoas que convivem nele, tornando-o um lugar aconchegante, mesmo sendo um ambiente de trabalho. Conheci muitas pessoas, cada um com seus objetivos, personalidades, ambições, sonhos e realizações expressos no "fazer ciência". Tanta gente que admiro e torço para que consigam tudo que almejam onde quer que estejam e que passaram (ou estão até hoje) pelo LABODE. Fiz amigos para a vida inteira e tenho a certeza de que, assim como eu, levam o aprendizado, a formação e o espírito da nossa convivência consigo. Realmente encerro esse ciclo com muita saudade, mas desejando que os novos pesquisadores em formaçãoque vierem a integrar o LABODE sejam tão felizes quanto eu. Peço desculpas por não poder citara todos e todas que fizeram parte do time do laboratório, porém preciso agradecer nominalmenteaos *Local Legends*: Júlia Meireles, Igor, Aline Copola, Íria, Alinne Costa, Juliano, Clara, Mari, Iago Lage, Júlia Helena, Nayara, Amanda Moreira, Renato, Amanda Senna, Ricardo, Carol Galinari, Raquel, Samira, Luiza, Cristiane Toledo, Aline Martins e ao Bruno (este último em

especial pelas oportunidades e parcerias científicas e de ensino). Agradeço também ao Chico (carinhosamente chamado de Francis) pela ajuda de sempre e pela ida aos "cafés e pasteis".

Ainda sobre o laboratório, eu necessito fazer uma menção honrosa e agradecer à Júlia Meireles. Não há palavras que consigam expressar o que foi ter o privilégio e a oportunidade de trabalhar com uma pessoa tão doce, amável, compreensiva, parceira, feliz, alegre, mas sem deixara competência em segundo plano. Como costumamos sempre dizer, nossos Lattes são basicamente cópias um do outros até meados de 2014, tamanha nossa afinidade científica, desde quando começamos a dar nossos primeiros passos em nossas aventuras pela ciência. Com quemmais eu iria querer começar a fazer um protocolo de hibridização *in situ* (vale dizer aqui que erao protocolo de uma semana, com mais de 5 horas de dedicação diárias a esse protocolo) às 16 horas e adentrar a noite?! E o Sincicinho criado às 21 horas no LABODE? Que e quantos momentos! Mesmo que hoje nossas atividades de pesquisa sejam em áreas distintas, nós nunca nos abandonamos (e nem iremos), pois conseguimos colaborar, desenvolver e participar em outros projetos, sempre com nossa sinergia única. Como dizem, nós desenvolvemos tamanha afinidade profissional e pessoal, que tem dias que a gente consegue falar de um punhado de assuntos, mesclando vida profissional e pessoal, em nosso dialeto e em linhas de raciocínio peculiares. Obrigado por permitir que eu crescesse ao seu lado. Eu queria deixar aqui registradoa minha admiração e orgulho pela pessoa que você tem se tornado a cada dia. Obrigado por ser uma das melhores amigas que a vida me trouxe. Quero expressar aqui todo meu amor a você e dizer que eu vou sentir muita falta do nosso convívio diário. Desejo todo sucesso para a gente e como você diz: "tamo junto, Ryan (sim, preciso registrar que foi você quem começou a me chamar assim e adotou a pronúncia internacional do meu nome)"!

À toda Equipe do CBEIH, que dividiu comigo esses últimos quatro anos e que conseguiu perseverar e entregar ciência de qualidade em meio a uma pandemia. Companheiros de muitas reuniões presenciais e virtuais, quero registrar o quanto aprendi e amadureci nessa equipe multidisciplinar. Obrigado por abraçarem muitas de minhas ideias envolvendo o mundo da biologia molecular e permitir estabelecer tantas conexões e colaborações tão frutíferas. Agradeço, especialmente à Clara, que mais do que uma aluna de iniciação científica, a qual tiveoportunidade de orientar, tornou-se uma amiga próxima e por quem tenho imenso carinho, podendo contribuir e participar de alguma forma para seu crescimento profissional. Obrigado porser meu braço direito durante a realização desse processo de doutorado. Tenho muita admiraçãopela aquisição de sua independência quanto pesquisadora e como você ganhou não apenas meu respeito, mas de toda a equipe. Que seu brilhantismo continue com você, independente do caminho que você escolher. Em suas palavras: "conte comigo para tudo!". Agradeço à Kelly,

sempre solícita, eficiente, disposta a resolver, com extrema maestria, conflitos e impedimentos referentes à realização do projeto, conseguindo sempre soluções benéficas e diferenciais, sem perder a irreverência e bom humor. Agradeço também à Gabi (referida por mim, simplesmente, como “a pessoa mais inteligente que eu conheço”), à Amanda Moreira (que abraçou a ideia queeu propus para seu mestrado e que o executou com dedicação e competência inigualáveis), ao Davi (que chegou no final do projeto, mas que trouxe frescor científico para a equipe), ao Fabiano(que é um exemplo de profissionalismo, divide a mesma data de aniversário comigo e certa vez me disse que eu deveria sempre confiar em mim mesmo, afinal eu era um bom profissional), e àsaudosa Jacque (que sempre foi sinônimo de clareza, objetividade e serenidade).

À toda equipe da Escola Estadual Professor Affonso Neves, que desde 2019, me recebeude maneira maravilhosa. Eu afirmo veementemente que muito de minha perspectiva de vida modificou-se após meu ingresso no ensino público de educação básica. Sou parte do corpo docente, porém os estudantes não sabem o quanto eles me ensinam. Sou grato pelas demonstrações de afeto que recebo deles diariamente. Apesar dos desafios que a educação enfrenta, eu não poderia ter escolhido melhor escola para trabalhar. Agradeço à Adriana, que desde a minha chegada à escola, sempre foi compreensiva (e isso se estende a todos os outros funcionários da escola), conseguindo ajustar meus horários para que fossem compatíveis com minhas outras atividades, além da competência em executar com perfeição as funções pedagógicas a administrativas. Suas risadas de Dona Álvara preenchem a escola! Agradeço ao Felipe, que foi afinidade a segunda vista, porque ele era bem reservado; contudo, hoje, a gente se diverte, canta, dança, basicamente somos memes ambulantes, mas que eu admiro demais comoprofissional. Como eu sempre falo, eu não poderia partilhar a Ciências/Biologia melhor do que com a Ana Paula; muito obrigado por ser uma professora com visão moderna, preocupação como bem-estar de todos, pela dedicação, seriedade, pela ‘zuêra’ e risadas de sempre. Obrigado ao Breno e Mariete pela calmaria e sensatez (a gente aprende isso no curso de História?), ao Fábio por colocar sempre os estudantes em primeiro lugar, à Kelly por sempre me oferecer doces e que,segundo os estudantes, detém o título de mais brava (fico feliz com o 2º lugar nesse caso), e à Letícia, cuja energia jovial, teatral e dançarina vigora e contagia a todos. Faço uma menção honrosa ao Ariel, que me acolheu, me ajudou quando ingressei e por quem tenho tamanha admiração e tive a oportunidade de desenvolver outros trabalhos e parcerias.

À Escola Municipal Professor Wancleber Pacheco, da qual passei a fazer parte em 2022,e que me recebeu de braços abertos. Ter a oportunidade de lecionar para a EJA é uma experiência diferencial, porque além de conteúdo, discute-se trajetórias e histórias de vida diversas, as quaisme levam a refletir e agregam grande aprendizado para mim. Agradeço à Conceição que foi

solícita desde o primeiro contato, à Grace, Walquíria e Ilda pelo modo como conduzem a escola,aos professores e já amigos Luciana, Cristiano, Maurílio, Cristina, Júnia, Maria e Ildete e aos demais funcionários Eduardo, Gustavo, Gabriel e Fernando. Quero reforçar o agradecimento a todos meus alunos, que tem (ou tiveram) aula comigo, desde os que frequentam a educação básicaaté os de pós-graduação; vocês são fontes de inspiração! Aproveito o ensejo para agradecer especialmente meus alunos da graduação de Medicina Veterinária da UFMG Iaritza, Pedro, Luiza, Samuel, André e Clara por terem criado e seguido criando conteúdo para o perfil do Instagram @fofoca_embriologica, que surgiu de um trabalho da disciplina de Embriologia e quegerou uma parceria maravilhosa entre nós.

Ao *ACS Student Chapter* da UFMG pela oportunidade de trabalhar outra vertente tão importante da ciência que é a divulgação científica. Agradeço enormemente ao convite do Gustavo e do Matheus para elaboramos o divulgAÇÃO. Percebi durante a concepção desses eventos que eu me realizo editando documentos e pessoas, não é mesmo, Ana Luiza Dornas?! OdivulgAÇÃO me colocou em contato com tantas pessoas diferentes, com formações distintas e experiências variadas, que me despertaram novos olhares acerca da imensidão científica. Agradeço aos Professores e Orientadores do *Chapter* Prof. Diogo Vidal e Profa. Roberta Corrêa,bem como aos integrantes Gustavo, Matheus, Ana Luiza, Luely Wesley, Amanda, Clara, Mariana, Leonardo, Isabelle, Marcus Vinícius, Rebecca e Daiane. Faço um adendo de gratidão àProfa. Roberta com quem dividi a orientação do Gustavo Elyseu, quando fui coorientador em umtrabalho de TCC da Química da UFMG.

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Existe um ditado que diz: “amigos são a família que podemos escolher” e que é potencializado quando você pode estender essa escolha ao campo pessoal e profissional. Percebihá pouco tempo que muitos dos meus amigos íntimos são colaboradores de muitos dos trabalhosdos quais desenvolvo e participo, incluído aos capítulos desse trabalho de tese. Agradeço à Prica(ou PriSCila) pela sabedoria e aconselhamentos, agradeço pelas experiências alimentícias em visitas gastronômicas de luxo, pelas fotos que só ela sabe tirar. Eu amo seu batom vermelho e seus eternos 31 anos, que vamos comemorar para sempre. Nunca vou me esquecer de nossa *vibe*com o carrinho no ICB cantarolando a vinheta de abertura de Fantástico Mundo de Bob ou o icônico momento “eu amo raspas, raspas de limão!”. Como você sempre fala: você capta minhaalma, minha essência. Obrigado por permitir que eu me expresse em minha totalidade quando estou em sua companhia! Você é fonte de amor, compreensão, ternura e inspiração; exemplo demulher! Ao Gustavo quero agradecer pelo companheirismo, pela compreensão, pela isenção de julgamento, pela paciência e pelo incentivo. Você sempre diz que, de alguma forma, eu sou fontede admiração e inspiração para você, entretanto eu queria deixar registrado que é totalmente recíproco. Quando recordo de muitos momentos divertidos em minha vida, você sempre está neles! Você faz parte da minha trajetória e temos muita história (e teremos muitas outras) para contar. Obrigado pela confiança quanto à orientação de seu TCC. Obrigado por me ajudar a dar nomes alternativos para os campeões do LoL, isso torna o jogo muito mais interessante. Obrigadopor trazer alegria à minha vida (mesmo você sendo sistemático e teimoso, às vezes) e por sua sinceridade e honestidade! Aos meus dois amigos André e Carlos, hoje referências em bioinformática, mas que nos conhecemos desde nossa graduação. Agradeço ao André por ser meu referencial de discernimento e racionalidade. Sempre que eu preciso de leituras precisas e isenção sobre situações, basta te consultar para chegarmos a um nível de sensatez indiscutível. Independente de agora você ser australiano e estarmos em fusos distintos, eu nunca vou deixar de me orgulhar e me sentir privilegiado por ter um amigo grandioso como você. Obrigado pela empatia, compreensão e cumplicidade mútuas! Ao Carlos, agradeço por sua consideração, paciência, ensinar sobre lidar com problemas e sua positividade. Nossos hobbies em comum nostornam muito próximos! Adoro sua empolgação, devoção e dedicação quando volta sua energiapara alguma coisa que gosta, isso é inspirador. Obrigado por me proporcionar tantas discussões nerds e se fazer presente sempre que possível!

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“O passado é apenas o futuro com as luzes acesas.”

Trecho (em tradução livre) retirado da música *Baby, Come On* da Banda +44.

RESUMO

Para a União Internacional para Conservação da Natureza (UICN), as invasões biológicas são a segunda maior causa de perda da biodiversidade no planeta. Algumas dessas espécies invasoras aquáticas, como o bivalve *Limnoperna fortunei* e o hidrozoário *Cordylophora caspia* destacam-se por causar grande impacto ambiental e econômico em plantas industriais, devido à sua capacidade em se aderirem fortemente a tubulações e trocadores de calor, processo denominado bioincrustação. Esse processo é complexo e, geralmente, iniciado pela formação de um filme primário composto por microrganismos, que além de contribuir para adesão de invertebrados, podem também compor a lama ferruginosa. A detecção e identificação rápida de um invasor é essencial para que ações de manejo sejam imediatas. Contudo, a identificação morfológica desses organismos nem sempre é uma tarefa fácil e pode gerar relatórios errôneos e controversos sobre a presença de tais organismos no ambiente. Neste contexto, a biologia molecular compõe um conjunto de técnicas, com alto poder de resolução, que, em conjunto com a identificação morfológica, conferem robustez à detecção e posterior controle da bioincrustação. Desta forma, o objetivo do presente trabalho foi propor o uso de ferramentas moleculares que auxiliem a detecção e controle de espécies bioincrustantes de hidrelétricas. Relativo à microincrustação, um estudo anterior do nosso grupo revelou uma presença significativa de ferrobactérias na lama ferruginosa. Assim, no presente trabalho, a detecção deste grupo, bem como de suas bioassinaturas (*stalks e sheaths)*, foram detectados em amostras de sedimento coletadas em outros pontos de uma hidrelétrica. Além disso, este trabalho reportou a presença inédita do macroincrustante *C. caspia* em duas hidrelétricas da região centro-oeste e sudeste do Brasil, bem como gerou as primeiras sequências genéticas desse invasor em território brasileiro. A coocorrência de *C. caspia* e *L. fortunei* deve ser detectada precocemente, uma vez que os prejuízos ambientais e econômicos decorrentes da presença desses dois invasores podem ser agravantes para hidrelétricas, perturbando serviços essenciais como a geração e distribuição de energia elétrica. Entretanto, é válido ressaltar que, tão importante quanto identificar os danos causados pelas bioincrustações, é o reconhecimento do potencial biotecnológico desses organismos micro e macroincrustantes, que servem como fonte de inspiração para criação de produtos e tecnologias com diversas aplicações, que direcionam, inclusive, maneiras de como reutilizar o material biológico oriundo deste processo.

Palavras-chave: bioincrustação, biologia molecular, espécies invasoras, detecção rápida, hidrelétrica

ABSTRACT

For the International Union for Conservation of Nature (IUCN), biological invasions are the second biggest cause of biodiversity loss on the planet. Some of these aquatic invasive species, such as the bivalve *Limnoperna fortunei* and the hydrozoan *Cordylophora caspia*, can cause great environmental and economic impact on industrial plants due to their ability to strongly adhere to pipes and heat exchangers, a process called biofouling. This process is complex and generally starts with the formation of a primary film composed of microorganisms, which besides contributing to the adhesion of invertebrates, can also compose the microbial slime. The early detection and rapid identification of fouling organisms are essential for management actions to be immediate. However, the morphological identification of these organisms is not always an easy task and can generate erroneous and controversial reports about the presence of such organisms in the environment. In this context, molecular biology composes a set of techniques, with high resolving power, which together with the morphological identification, give robustness against biofouling. Thus, the objective of the present study was to propose the use of molecular tools to assist the detection and control of biofouling species of hydroelectric plants. Regarding microfouling, stalks-type biosignatures, produced by iron-oxidizing bacteria belonging to the microbial slime community, were found in sediment samples collected in a hydroelectric plant. In addition, this work has firstly reported the presence of *C. caspia* in hydroelectric plants in the Brazil central-west and southeast regions, as well as generating the first genetic sequences of this invader in Brazilian territory. The co-occurrence of *C. caspia* and *L. fortunei* must be carefully observed, since the environmental and economic losses resulting from the presence of these two invaders can be aggravating for hydroelectric plants, disrupting essential services such as the generation and distribution of electricity. However, it is worth noting that, as important as identifying the damage caused by biofouling, it is the recognition of the biotechnological potential of these fouling organisms, which serve as a source of inspiration for the creation of products and technologies with diverse applications, even ways on how to treat biofouling itself.

Keywords: biofouling, molecular tools, invasive species, early detection, hydropower plant.

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LISTA DE ABREVIAÇÕES E SIGLAS

µL: microlitro

µm: micrômetro

ANEEL: Agência Nacional de Energia Elétrica

bp/ pb: *base pairs*/ pares de base

BSA: *bovine serum albumin*

CBEIH: Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas

CEMIG: Companhia Energética de Minas Gerais

cm: centímetro

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico

COI: Citocromo C Oxidase Subunidade I

CRISPR: Repetições Palindrômicas Curtas Agrupadas e Regularmente Interespaçadas

CS: *cave sediment*

DMSO: *dimethylsulfoxide*

DNA: ácido desoxirribonucleico Dopa:

3,4- dihydroxyphenyl-a-alanine

DRRI: Detecção Rápida e Resposta Imediata

EDS: *Energy Dispersive X-ray Spectroscopy*

EPS: exopolissacarídeo

Fe: ferro/ *iron*

FeOB: Bactérias oxidadoras de ferro/ *iron-oxidizing bacteria*

FS: *filter sediment*

HEM: *microfouling*

LAMP-PCR: Amplificação Isotérmica da Reação em Cadeia da Polimerase

min: minuto

MS: *microbial slime*

NCBI: *National Center for Biotechnology Information*

ng: nanograma

nm: nanômetro

º C: graus Celsius

OTU: Unidade Taxonômica Operacional

P&D: Pesquisa e Desenvolvimento

PCR: Reação em Cadeia da Polimerase

RFLP: *Restriction Fragment Length Polymorphism*

RNA: ácido ribonucleico

RNAr: ácido ribonucleico ribossomal

RS: *rock sediment*

s: segundo

SEM: *Scanning Electron Microscopy*

SISBIO: Sistema de Autorização e Informação em Biodiversidade

SisGen: Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado

SRA: *Sequence Read Archive*

UEMG: Universidade do Estado de Minas Gerais

UFMG: Universidade Federal de Minas Gerais

UHE: Usina Hidrelétrica

UICN: União Internacional para Conservação da Natureza

ESTRUTURA DA TESE

Esta tese foi organizada de forma a conter uma introdução geral, abordando os principais temas propostos, seguida dos objetivos gerais e específicos. A metodologia, resultados e discussão serão apresentados na forma de três capítulos, sendo o primeiro referente à microincrustação, o segundo referente ao macroincrustante invasor *Cordylophora caspia*, e o terceiro referente ao macroincrustante invasor *Limnoperna fortunei*, este último subdividido em três tópicos. Para finalizar, serão apresentados os trabalhos complementares desenvolvidos durante o desenvolvimento e até a conclusão desta tese.

A relação de revistas bem como o *status* referente a cada um dos artigos estão descritos conforme a tabela a seguir:

Capítulo	**Manuscrito**	**Periódico**	***Status***
1	Diversity and distribution of iron-oxidizing bacteria belonging to Gallionellaceae in different sites of a hydroelectric power plant	Journal of Industrial Microbiology and Biotechnology (ISSN: 1476-5535)	Submetido
2	Cordylophora caspia (Pallas 1771) (Cnidaria, Hydrozoa) in Brazil: a review of the current scenario with new records, evolutionary inferences, and perspectives	Biological Invasions (ISSN: 1573-1464)	Submetido
3.1	Genetic and functional repertoires of *Limnoperna fortunei* (Dunker, 1857) (Mollusca, Mytilidae): a review on the use of molecular techniques for the detection and control of the golden mussel	Hydrobiologia (ISSN: 1573-5117)	Publicado
3.2	A Case for the Continued Study of the Golden Mussel Invasion of Brazil: Efficient Detection and Containment of *Limnoperna fortunei* (Dunker, 1857) Dispersion Involves Multiple Approaches and Different Actors	Oceanography & Fisheries Open Access Journal (ISSN: 2476-0536)	Publicado
3.3	Laboratory standardization of the Loop-mediated Isothermal Amplification (LAMP) assay for detection of *Limnoperna fortunei* (Dunker 1857)	A ser decidido	A ser finalizado

SUMÁRIO

1. Introdução

1.1. Bioincrustação: micro e macroincrustação

Bioincrustação, bioacumulação ou *biofouling* é um processo no qual os organismos são capazes de se fixar em diferentes superfícies e materiais (Kanematsu & Barry, 2020). Segundo Epstein (1981), o termo bioincrustação surgiu da observação da deposição indesejada de camadas microbianas, denominadas biofilme microbiano, sobre superfícies de trocadores de calor, componentes importantes do sistema de resfriamento de indústrias. A bioincrustação é categorizada em dois tipos: microincrustação, relacionada à formação do biofilme microbiano, e macroincrustação, referindo-se à fixação de organismos multicelulares, como macroinvertebrados e plantas, alguns dos quais são considerados espécies invasoras (Sanders & Maxwell, 1983; Dobretsov et al., 2013; Kanematsu & Barry, 2020). Por ser um processo complexo, com influência abiótica (matéria inorgânica, como argila, substâncias húmicas, sílica, e orgânica, como glicoproteínas, óleo, proteínas) e biótica (microrganismos e/ou macrorganismos), a bioincrustação forma-se sucessivamente em estágios paralelos ou sobrepostos (Flemming, 2002; Dobretsov et al., 2006; Qian et al., 2007; Komlenic, 2010; Lu et al., 2016).

No início do processo de microincrustação, a superfície é naturalmente revestida por componentes inorgânicos (íons, sais) e orgânicos (componentes ricos em carbono, proteínas) presentes na água, formando um filme condicionante (Schneider & Marshall, 1994; Siboni et al., 2009). Posteriormente, ocorre a colonização primária de microrganismos, que começam a sintetizar fatores quimiotáticos e a secretar a matriz exopolissacarídica (EPS), iniciando um processo sucessional até se tornar um biofilme maduro e susceptível à colonização de organismos macroscópicos (Taylor et al., 1997; Donlan, 2002; Thome et al., 2012; Kanematsu & Barry, 2020) (Figura 1). A organização, composição e manutenção da estrutura da comunidade bioincrustante conferem aos organismos residentes inúmeras vantagens, como a facilidade de trocas de metabólitos, elaboração de respostas rápidas diante de adversidades ambientais e frente a tratamentos anti-incrustantes (Crabbé et al., 2019; Yan e Bassler, 2019).

Figura 1. Sucessão do processo de bioincrustação. Moléculas orgânicas, de escala nanométrica, são carreadas pela água e revestem a superfície limpa, dentro de segundos, formando o filme condicionante. Decorridos alguns minutos, microrganismos, como bactérias e diatomáceas, começam a se aderir sobre o filme condicionante e formam o biofilme. Organismos macroincrustantes podem se assentar sobre o biofilme e, decorridos dias, ou até mesmo, meses, essa comunidade bioincrustante passa a ocupar um maior volume e torna-se madura, repleta de organismos micro e macroincrustantes que interagem entre si. Figura adaptada de Kirschner & Brennan, 2012.

A bioincrustação é responsável por causar substanciais prejuízos econômicos a plantas industriais, como observado em hidrelétricas (Almeida et al., 2016). A obstrução de trocadores de calor, por exemplo, resulta no aumento da resistência à transferência de energia entre o óleo lubrificante da turbina e a água circulante, impedindo seu resfriamento (Andrewartha et al., 2008; Frota et al., 2014). Como consequência, ocorre a parada de máquinas para que seja realizada a remoção dos agentes incrustantes e de seus resíduos biológicos, resultando em perda de geração de eletricidade (Almeida et al., 2016; Silva et al., 2016). Além destes prejuízos, esses organismos possuem o potencial de promover a biocorrosão de superfícies, cujos processos metabólicos produzem substâncias como ácidos orgânicos, que desgastam tal superfície, modificando-a (Coetser & Cloete, 2005; Portella et al., 2009).

1.2. Estratégias anti-incrustação

Ao longo dos anos, acompanhado por uma maior compreensão acerca da estrutura morfofuncional do processo de bioincrustação, várias contramedidas e tratamentos anti-incrustantes foram propostos para mitigar os fenômenos de incrustação observados em plantas

industriais, como descrito para sistemas de água de resfriamento em usinas hidrelétricas e de dessalinização de água potável (Fusetani, 2004; Dürr & Watson, 2010; Henderson, 2010; Cristiani & Perboni, 2014; Frota et al., 2014). As estratégias anti-incrustantes podem ser naturais e industriais (que incluem abordagens computacionais, de engenharia, métodos físicos e químicos) cujas ações são elaboradas para controlar a micro e a macroincrustação, sendo esta última mais complexa e, portanto, mais difícil de controlar (Bixler & Bhushan, 2012; Kirschner & Brennan, 2012; Kanematsu & Barry, 2020).

O lançamento de produtos e/ou tecnologias anti-incrustantes no ambiente enfrentam diversos desafios que variam desde a eficiência em eliminar a bioincrustação em si até questões ambientais complexas. A instalação das plantas industriais ocorre em locais que afetam atividades de vários segmentos sociais, como é o caso dos reservatórios de hidrelétricas, cuja água é utilizada não somente para geração de eletricidade, mas também para abastecimento da população e realização de atividades agropecuárias, tornando-se um fator complicador para a diluição desses produtos (Silva et al., 2009). Desde o início de 2008, um número crescente de países ratificou um tratado internacional para proibir a aplicação de revestimentos anti-incrustantes baseados em compostos organoestânicos (por exemplo, tributilestanho e trifenilestanho). Como resultado, a demanda por compostos e tecnologias anti-incrustantes ecologicamente corretos, não tóxicos ou de baixa toxicidade (agentes anti-incrustantes “verdes”) tornou-se uma realidade urgente (Qian et al., 2009; Qian et al., 2015).

Naturalmente, diversos organismos apresentam mecanismos de prevenção à bioincrustação sobre sua superfície corporal, como os equinodermos (e outros invertebrados), que possuem a epiderme recoberta por uma fina cutícula de onde irradiam fibrilas voltadas para o meio externo e que, juntamente à produção de proteoglicano de sulfato de condroitina, conferem proteção contra a microincrustação (McKenzie & Grigolava, 1996; Bavington et al., 2004). O reconhecimento e descrição de tais mecanismos encontrados na natureza podem subsidiar a criação de produtos anti-incrustantes com aplicações industriais, reduzindo ao máximo os danos colaterais ao meio ambiente, como observados em compostos baseados em ácidos graxos, isolados da alga vermelha *Laurencia sp.* (Umezawa et al. 2014), e extratos a base de terpenóides retirados da esponja *Acanthella cavernosa* Dendy 1922 (Xu et al. 2012).

Industrialmente, os principais métodos utilizados para controlar a bioincrustação são físicos e/ou químicos, que se subdividem em metodologias reativas ou proativas (Cataldo, 2002; Netto, 2011). Métodos reativos são aqueles adotados após a instauração e convívio com o fenômeno bioincrustante na planta industrial, dentre os quais se destacam aplicações de choques térmicos no sistema, remoção mecânica e utilização de água pressurizada para retirada dos

organismos. Os proativos são os métodos preventivos ou que não toleram de forma alguma o convívio com a bioincrustação, já que paradas mais prolongadas do maquinário não seriam viáveis para realização de sua limpeza. Nesse caso, os métodos empregados são físicos: acústicos, eletromagnéticos, que variem a disponibilidade de oxigênio, gerando ambientes anóxicos e hipóxicos, e tratamento com luz ultravioleta; e químicos: cloro gasoso, dióxido de cloro, dicloroisocianurato de sódio, hidróxido de sódio, injeção de ozônio, pinturas anti-incrustantes e sulfato de cobre (Filippo, 2003; Giordani et al., 2005; LeBlanc et al., 2007; Wang et al., 2012). Vale ressaltar que a escolha de um ou de combinações dentre esses vários métodos deve ser avaliada quanto a compatibilidade entre produtos e os materiais utilizados na construção das instalações, além de estimar custos, que, por diversas vezes, mostram-se bastante elevados, inviabilizando a aplicação de tal tratamento (Netto, 2011). Atualmente, áreas envolvendo computação, engenharia, matemática e robótica vêm desenvolvendo tecnologias que empregam microrrobôs capazes de remover biofilmes dos sistemas industriais (Albitar et al.; 2016; Fan et al., 2018; Tian et al., 2020).

Por outro lado, o conhecimento sobre a biologia e ecologia dos organismos bioincrustantes pode auxiliar a delinear estratégias eficientes para sua mitigação (Hashimoto et al., 2012; Harrington et al.,2018; Renner-Rao et al., 2019). De fato, pesquisadores, institutos e empresas mostram-se cada vez mais interessados em descobrir e desenvolver tecnologias, patentes e produtos a partir da observação minuciosa do processo de bioincrustação (Gademann, 2007; Holm, 2012; Martinez et al., 2020).

1.3. Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas (CBEIH)

Criado em 2010, o Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas (CBEIH)[1] é fruto do P&D (Pesquisa e Desenvolvimento) ANEEL (Agência Nacional de Energia Elétrica) /CEMIG (Companhia Energética de Minas Gerais) GT-343 (2010-2016) e ganhou continuidade em 2018, com o P&D ANEEL/CEMIG GT-604. Formado por uma equipe multidisciplinar, o CBEIH baseia sua metodologia de pesquisa na prevenção, detecção e controle de organismos invasores, apoiado na interação entre diferentes áreas de conhecimento, entre as quais se destacam a bioengenharia, a modelagem e o monitoramento ambiental (Figura 2).

[1] www.cbeih.org

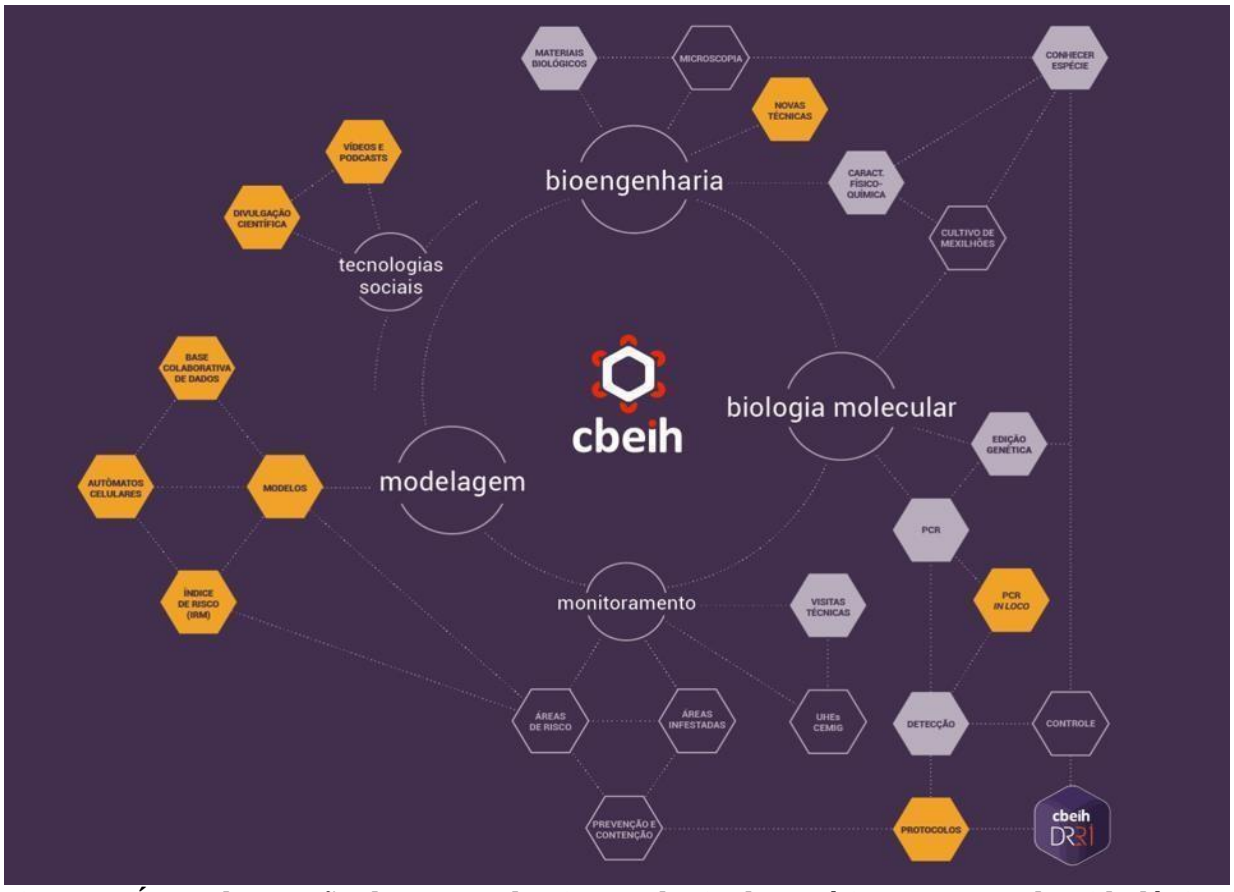

Figura 2. Áreas de atuação do Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas (CBEIH). Formado por uma equipe multidisciplinar, o CBEIH dedica-se a estudar aspectos ambientais e econômicos decorrentes da presença de espécies bioincrustantes em hidrelétricas. A atuação do CBEIH pauta-se na integração de múltiplas áreas do conhecimento, entre as quais se destacam a bioengenharia, a biologia molecular, a modelagem e o monitoramento ambientais. O CBEIH foi criado pelo P&D ANEEL/CEMIG GT-343 e ganhou continuidade, em 2018, com o P&D ANEEL/CEMIG GT-604. Fonte da imagem: CBEIH.

As invasões biológicas provocam mudanças profundas no ecossistema, afetando tanto as relações entre os seus diversos componentes quanto à disponibilidade de recursos naturais utilizados pelo homem (Boltovskoy & Correa, 2015). O processo de invasão pode ser dividido em uma série de etapas, sendo que em cada uma delas existem barreiras que precisam ser superadas para que uma espécie ou população passe para a próxima etapa. Resumidamente, cada etapa está associada à superação de uma barreira, que por sua vez está atrelada a ações de manejo e intervenções adequadas (Blackburn et al., 2011). A detecção rápida da chegada de um invasor é essencial, já que permite o controle de subpopulações enquanto ainda são pequenas o suficiente para serem erradicadas e faz com que ações de manejo sejam imediatas, além de impedir que aquele ambiente sirva como vetor para novas infestações (Do Sul, 2007; Vander Zanden &

Olden, 2008). No entanto, a efetiva capacidade de monitorar estes organismos é ainda limitada e a consolidação de um protocolo de monitoramento inteligente, integrado a um sistema de alerta, torna-se uma estratégia eficiente e barata, que possivelmente irá diminuir os impactos ecológicos e econômicos.

No contexto das invasões biológicas, desde sua fundação até o presente momento, a espécie invasora carro-chefe dos estudos do CBEIH é o mexilhão-dourado, *Limnoperna fortunei* (Dunker 1857). Nativo do sudeste asiático, esta espécie invadiu a América do Sul na década de 1990, apresentando incrível capacidade bioincrustante, especialmente em usinas hidrelétricas (Darrigran & Pastorino, 2004). Visto que a detecção rápida de *L. fortunei* é uma possível maneira de mitigar o processo de invasão, o CBEIH criou o "Programa de Detecção Rápida e Resposta Imediata" (DRRI), baseado no programa de *Early Detection* (Hosler, 2011) do *Bureau of Reclamation*, instituto responsável pelo combate às espécies invasoras nos Estados Unidos. O DRRI apresenta uma combinação de técnicas, dentre elas a tríade laboratorial que utiliza estereomicroscopia com luz polarizada, o analisador de partículas em fluxo (FlowCam®) e o uso do DNA *barcoding* (Pie et al., 2006), para a identificação de larvas do mexilhão-dourado em amostras de água (dados ainda não publicados) (Figura 3).

Figura 3. Programa de Detecção Rápida e Resposta Imediata (DRRI) desenvolvido pelo Centro de Bioengenharia de Espécies Invasoras de Hidrelétricas (CBEIH). O DRRI é composto por etapas e abordagens que permitem a detecção de espécies invasoras no ambiente, tão cedo quanto possível. Inicia-se por um monitoramento estratégico em campo, seguido por ensaios laboratoriais, que combinam a estereomicroscopia com luz polarizada, o analisador de partículas em fluxo (FlowCam®) e a biologia molecular para confirmar a presença de determinado invasor; e, confirmada sua presença, aciona sistemas de alerta integrados, para que as estratégias de prevenção, controle e/ou combate sejam imediatamente aplicadas, na tentativa de frear um novo processo de invasão em determinado local ou manter informações acerca de locais já invadidos. Fonte da imagem: CBEIH.

Atualmente, além do mexilhão-dourado, o CBEIH expandiu seus estudos para outras espécies que causam bioincrustação em hidrelétricas e que podem estar (ou não) associadas a *L. fortunei*. São elas: o cnidário invasor *Cordylophora cáspia* (Pallas 1771), hidrozoário nativo dos mares Cáspio e Negro, e os microrganismos que compõem a lama ferruginosa, sendo ambos agentes biológicos que também podem se incrustar em tubulações e sistemas de resfriamento de hidrelétricas.

1.4. Microincrustação decorrente da formação da lama ferruginosa

Além de monitorar as características físico-químicas de um reservatório, detectar sua diversidade biológica também é de grande relevância, já que contribui para a variabilidade genotípica e a atividade metabólica ali existentes. Sobre a diversidade microbiana em plantas industriais, é bem conhecido que algumas bactérias se estruturam como um biofilme, resultando em incrustações que atingem captações de água e os próprios sistemas de distribuição de uma usina (Frota, 2013). Portanto, a detecção quantitativa e qualitativa da comunidade microbiana a partir de amostras ambientais é essencial para entender a dinâmica de um determinado ecossistema (Prakash & Taylor, 2012). Contudo, não é possível determinar a diversidade microbiana de um ambiente utilizando apenas técnicas dependentes de cultivo (Rondon et al., 2000). Como alternativa, abordagens envolvendo técnicas independentes de cultivo, como a metagenômica, nos permite acessar todo o repertório genético de microrganismos de um determinado ambiente (Pace, 1997).

Sob o ponto de vista econômico, uma das situações críticas que afetam o funcionamento pleno de uma hidrelétrica é a microincrustação, que pode ser caracterizada pela formação da lama ferruginosa, composta por biofilme microbiano associada ao acúmulo de pequenas partículas de sedimentos transportadas pela água corrente no rio (de Lima et al., 2008). Vale ressaltar que a água do próprio reservatório, filtrada em equipamento autolimpante, é utilizada diretamente nos sistemas de resfriamento da hidrelétrica, como em radiadores e trocadores de calor. Portanto, as alterações pelas quais a água de um reservatório está sujeita, podem ocasionar em eutrofização e estratificação química e/ou térmica, o que gera aumento de matéria orgânica disponível para incrustar em materiais e equipamentos das usinas, formando o filme condicionante (Segundo et al., 1993). Essa água não é submetida a nenhum tipo de tratamento prévio, carreando, dessa forma, os componentes abióticos e bióticos para o interior desse sistema de resfriamento, onde se deposita sob a forma de biofilmes microbianos (Netto, 2011).

No Brasil, a lama ferruginosa figura como um relevante desafio em hidrelétricas, como por exemplo na Hidrelétrica de Irapé (UHE-Irapé), situada no Rio Jequitinhonha, no estado de Minas Gerais, que se deposita em trocadores de calor do sistema de resfriamento da usina (Figura 4) (Lima, 2009).

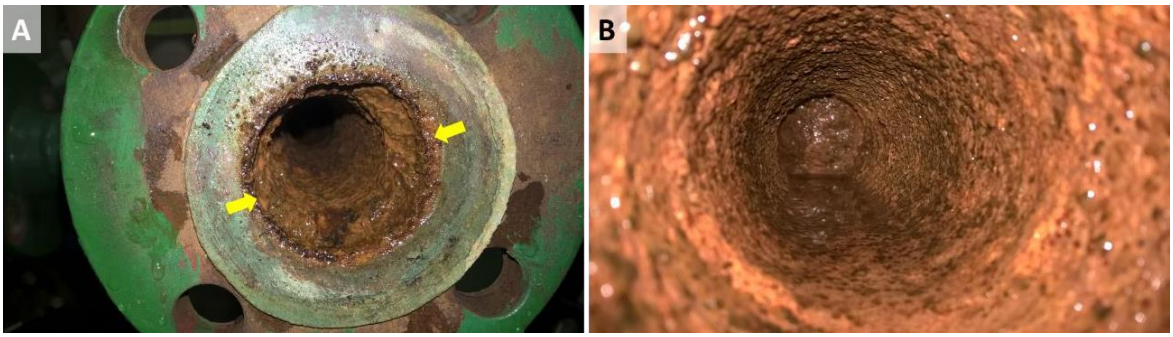

Figura 4. Deposição de lama ferruginosa na Usina Hidrelétrica de Irapé (UHE-Irapé). Em (A), microincrustação ocasionada pela associação entre o biofilme microbiano e o acúmulo de partículas de sedimentos carreados pela água do reservatório, formando o fenômeno conhecido como lama ferruginosa (setas), em trocadores de calor da UHE-Irapé (Minas Gerais, Brasil). Em (B), detalhamento da lama ferruginosa no interior do trocador de calor. Fotos: CBEIH.

No recente trabalho de nosso grupo, Reis et al. (2021) investigaram a diversidade microbiana em amostra de lama ferruginosa, coletada no trocador de calor; e sedimento, coletado do filtro de água industrial, ambos componentes do sistema de resfriamento da UHE-Irapé. Esta usina situa-se em uma região rica em ferro e enxofre (Mendonça et al., 2001; Lima, 2009). Foram feitas duas amostragens em ambos componentes. A lama ferruginosa da primeira coleta havia sido formada 30 dias após limpeza do trocador de calor, sobre o qual utilizou-se um composto baseado em tanino e água pressurizada; enquanto a lama ferruginosa da segunda coleta formou-se após 15 dias da limpeza do trocador de calor, sendo removida somente por meio de uso de água pressurizada. Os resultados obtidos nesse estudo revelaram uma assinatura específica na comunidade microbiana da lama ferruginosa com formação mais recente, indicando que os gêneros exclusivos *Thermomonas*, *Meiothermus* e *Symbiobacterium* e OTUs (traduzindo do inglês, Unidades Taxônomicas Operacionais) relacionadas à Bacteroidetes e Gallionellaceae poderiam ser os prováveis colonizadores primários do biofilme microbiano. Por outro lado, a comunidade microbiana presente no sedimento do filtro foi marcada pela ocorrência significativa de bactérias metanotróficas e redutoras de Fe.

Destaca-se nesse estudo a abundância de Gallionellaceae dentre as amostras coletadas (5% do número total de *reads*), sendo sua presença significativa na lama ferruginosa recentemente depositada. Além disso, dentre os membros da família Gallionellaceae, a maioria não foi classificada em nível de gênero (99% das *reads* relacionadas à Gallionellaceae). É bem conhecido que membros desta família desempenham um papel fundamental na oxidação do Fe em ambientes neutrofílicos e na formação da lama ferruginosa (Mulder e Deinema, 1981; Wang, 2011; Reis et al., 2014), embora a diversidade filogenética e propriedades ecológicas destes

membros ainda sejam pouco exploradas (Reis et al., 2014).

É válido ressaltar que a oxidação biótica do ferro pode ser conduzida pelas FeOB (bactérias oxidadoras de ferro) de maneira passiva (mudança do pH local e sorção físico-química passiva) ou ativa (conversão enzimática para óxido de ferro) (Fortin et al., 1998; Hanert, 2002; Widdel et al., 1993; Wang et al., 2009). É bem descrita a eficiência de FeOB acidofílicas na oxidação do ferro, aumentando a taxa de oxidação do ferro em quatro ordens de magnitude, quando comparada à oxidação abiótica (Søgaard et al., 2001). As FeOB neutrofílicas, por sua vez, têm sido menos estudadas devido às dificuldades associadas ao cultivo e manutenção das culturas (Edwards et al., 2004). Entretanto, estudos revelam que FeOB neutrofílicas são responsáveis por até 60% da oxidação total do Fe em comparação com a oxidação estritamente abiótica, revelando sua importância no ciclo do ferro em ambientes de pH neutro (Søgaard et al., 2001; Emerson & Moyer, 2002; Wang et al., 2009). Dentre as FeOB neutrofílicas, os gêneros *Gallionella*, *Sideroxydans* e *Leptothrix* são os predominantes no ambiente (Wang et al., 2009; Wang et al., 2011).

Associadas às metodologias metagenômicas, algumas bactérias da família Gallionellaceae, produzem bioassinaturas morfológicas, passíveis de serem reconhecidas por técnicas ultramicroscópicas, encontradas em sedimentos e, assim, corroboram com sua identificação em determinado ambiente. *Gallionella* e *Ferriphasellus*, este último também pertencente à Gallionellaceae, são gêneros conhecidos por produzir *stalks*, bioassinaturas inicialmente compostas por polímeros de carbono, organizados em numerosas fibras paralelas eletrondensas e que, posteriormente, são incrustadas por Fe e outros metais (Kato et al., 2014). As bioassinaturas promovem a fixação dessas bactérias ao sedimento, ajudam na captação e agregação de metais indispensáveis a seu metabolismo e auxiliam a manutenção da estrutura do biofilme (Kato et al., 2015). Estudos têm empregado o uso de dessas bioassinaturas como biomateriais para desenvolvimento de tecnologias, tal qual para a composição de baterias de lítio e até mesmo como pigmentos para tintas, esmaltes e vidrados (Hashimoto et al., 2012; Hashimoto et al., 2014). Dessa forma, a identificação dos membros da comunidade microbiana, bem como a determinação da riqueza e abundância das espécies que colonizam o biofilme, tornam-se o ponto de partida para traçar ações que minimizem fenômenos microinscrustantes, como observado pela deposição da lama ferruginosa.

1.5. Macroincrustação causada por espécies invasoras

1.5.1. *Cordylophora caspia*

Nativa dos Mares Cáspio e Negro, *Cordylophora caspia* é uma espécie de cnidário invasor, pertencente à Família Cordylophoridae, Ordem Anthoathecata, Classe Hydrozoa e apresenta distribuição global (Clarke, 1878; Wollschlager, 2011). Acredita-se que, assim como *L. fortunei*, esse hidrozoário invadiu novos ambientes transportado por águas de lastro, incluindo o Brasil (Haddad & Nakatani, 1996; Grohmann, 2008). Seu potencial invasor associa-se à competência de *C. caspia* em colonizar habitats de água doce, salgada e/ou estuários (Pucherelli et al., 2016), bem como sua capacidade de adaptar-se a condições ambientais diversas, como tolerância a alterações de salinidade, de pH e à poluição das águas (Hubschman, 1971; Folino, 1999; Gutierre, 2012).

Análises moleculares recentes, incluindo uma gama global de populações de *C. caspia* oriundas de habitats de água doce e salobra, resultaram em vários clados com forte correlação na tolerância à salinidade, levantando a hipótese de que o gênero *Cordylophora* abrigaria espécies crípticas, com diferentes preferências de habitat ou uma única espécie eurialina geneticamente diversa (Folino-Rorem et al. 2009; Pucherelli et al. 2016). Alguns autores acreditam quem *C. lacustris* habitaria a água doce e *C. caspia* ficaria restrita à água salobra (Folino, 1999; Schucher, 2004). Outros acreditam que as oito espécies listadas *albicola*, *annulata*, *caspia*, *dubia*, *inkermanica*, *lacustris*, *pusilla*, *whiteleggi* poderiam ser sinônimas, pertencentes a uma única categoria de espécie (Folino-Rorem et al., 2009). Baseado em inferências filogenéticas, quatro clados monofiléticos foram propostos: 1A, 1B, 2A e 2B, de modo que o clado 1 refere-se aos espécimes encontrados em água doce e o clado 2 em água salobra, de maneira não exclusiva, uma vez que espécimes do clado 1B foram encontrados habitando ambos os ambientes (Folino-Rorem et al., 2009). Devido a essas questões, é válido ressaltar a necessidade de revisão detalhada do gênero *Cordylophora* (Folino-Rorem et al. 2009; Folino-Rorem & Renken 2018).

Cordylophora caspia é colonial, cresce em vários tipos de substratos e possui dois tipos de pólipos: um para alimentação (hidrantes) e outro para reprodução (gonóforos) (Fulton, 1962). No ciclo de vida, *C. caspia* não possui o estágio medusóide, os embriões se desenvolvem nos gonóforos e se separam como plânulas ciliadas de natação livre, capazes de se fixar em um novo substrato e formar novas colônias (Roos, 1979). Agregados de populações de *C. caspia* podem causar danos a superfícies por meio da produção de uma densa rede de filamentos e pelo lançamento de produtos oriundos de seu metabolismo (Portella et al., 2009), além de promover obstruções de estruturas e tubulações em plantas industriais, como hidrelétricas (Folino-Rorem,

2015). Ecologicamente, em áreas invadidas, *C. caspia* parece ocupar o nicho de um predador colonial bentônico (Smith et al. 2002).

As colônias de *C. caspia* criam novos microhabitats para vários táxons, como algas, moluscos e crustáceos (Portella et al., 2009). Entretanto, a relação entre a manutenção destes microhabitats é dual, uma vez que este hidrozoário utiliza de seus tentáculos para neutralizar e alimentar-se desses mesmos organismos citados, incluindo larvas de moluscos invasores, como foi observado para o mexilhão quagga na América do Norte (Bij de Vaate et al., 2002; Pucherelli et al., 2016). Recentemente, essa dualidade foi também observada por da Silva Bertão et al. (2021) em relação ao mexilhão-dourado, de modo que *C. caspia* alimenta-se das larvas em estágio menor e serve de substrato para as larvas maiores, servindo de pontos de fixação para *L. fortunei* e impedindo que suas larvas sejam carregadas pela correnteza.

No Brasil, a associação entre *C. caspia* e *L. fortunei* foi observada na hidrelétrica Governador José Richa, no estado do Paraná, em que se avaliou, durante um ano, a colonização de placas de cimento por estes dois organismos invasores. Inicialmente, houve colonização das placas apenas pelo hidrozoário. Notou-se a colonização tardia por mexilhão-dourado em áreas expostas das placas causadas pela lixiviação prévia por *C. caspia* e também observou-se a adesão de *L. fortunei* sobre colônias de *C. caspia* anteriormente aderidas (Portella et al., 2009). Dado este panorama, é válido ressaltar que essa associação reforça a necessidade de uma rápida identificação de organismos invasores no ambiente, em especial quando os nichos ocupados por eles se sobrepõem e/ou se complementam, já que sua coexistência pode agravar ainda mais os danos ambientais e econômicos decorrentes desse processo de invasões biológicas bioincrustantes (Figura 5). da Silva Bertão et al. (2021) referem-se à essa associação de entre *C. caspia* e *L. fortunei* como mutualística, especialmente relacionados ao potencial incrustante que é danosa às instalações da hidrelétrica.

Figura 5. Associação entre as espécies invasoras bioincrustantes *Cordylophora caspia* e *Limnoperna fortunei* na Usina Hidrelétrica de São Simão (UHE-São Simão). Em (A), equipamentos da UHE-São Simão (divisa entre Minas Gerais e Goiás, Brasil) completamente incrustadas por *C. caspia* (cabeças de seta). Em (B), coocorrência entre os macroincrustantes invasores *C. caspia* e *L. fortunei* (setas). Fotos: SPIC Brasil.

1.5.2. *Limnoperna fortunei*

Limnoperna fortunei, o mexilhão-dourado, é um molusco bivalve pertencente à Família Mytilidae (Subclasse Pteriomorpha e Ordem Mytiloida), nativo do sudeste da Ásia (incluindo China e Coréia do Sul) e foi introduzido nos países da América do Sul provavelmente via águas de lastro de navios entre rotas comerciais estabelecidas entre esses continentes (Pastorino et al., 1993; Darrigran, 1995). Inicialmente introduzido no estuário do rio La Plata, da Argentina, em 1991, *L. fortunei s*e alastrou para mais de 5.000 km intracontinentais, em territórios de países como Bolívia, Brasil, Paraguai e Uruguai (Dreher *et al*., 2003; Darrigran & Pastorino, 2004). No Brasil, os registros mais recentes de *L. fortunei* reportaram a presença desse invasor na Bacia do São Francisco, na hidrelétrica de Sobradinho, situada na divisa entre os estados da Bahia e Pernambuco, em canais de transposição do rio São Francisco (Barbosa et al., 2016), e na hidrelétrica de Nova Ponte, na bacia do Rio Paranaíba, no estado de Minas Gerais (Boletim de Alerta nº 5, 2020).

Várias características do mexilhão-dourado garantiram seu sucesso como espécie invasora. *Limnoperna fortunei* possui duas valvas (conchas) constituídas de carbonato de cálcio, que conferem ao animal uma grande vantagem ecológica, já que, além de sustentar o corpo mole, o protege de agentes externos, como, por exemplo, predadores (Morton, 1977; Darrigran, 2002; Nakamura et al., 2014). São gonocóricos (com raros eventos de hermafroditismo constatados), possuem fecundação externa e desenvolvimento indireto, o que confere grande sucesso reprodutivo (Morton, 1977; Dei Tos et al., 2016). As larvas consistem em uma das principais formas de dispersão destes organismos invasores e atingem alta taxa de sucesso e adaptabilidade no ambiente invadido, devido à sua grande densidade no momento de dispersão e da falta de predadores e parasitos especializados enquanto adultos (Darrigran & Mansur, 2006). O indivíduo torna-se adulto após um período de aproximadamente um ano, atingindo, assim, a maturação sexual (Morton, 1982). As gônadas de *L. fortunei* apresentam estágios de maturação cíclicas que variam de acordo com a estação do ano, podendo ser divididos tais estágios em quatro: regressão, desova (*spawning*), desenvolvimento e regeneração. No entanto, apenas no verão, observou-se o estágio de desova em fêmeas de mexilhão-dourado, estação em que a temperatura da água se encontra mais elevada (Dei Tos et al., 2016).

O mexilhão-dourado tem causado impactos ambientais e econômicos para o Brasil. Dentre os impactos ambientais, *L. fortunei* tem facilitado, por exemplo, as florações tóxicas de cianobactérias (Cataldo *et al.*, 2012; Gazulha *et al.*, 2012). Economicamente, o mexilhão-dourado tem trazido grandes impactos para usinas hidrelétricas, principalmente por obstruir as tubulações dos sistemas de captação de água, gerando altos custos anuais de operação e manutenção (Nakano & Strayer, 2014). Os prejuízos causados por espécies invasoras nos Estados Unidos são estimados em 400 bilhões de reais e, no Brasil, até 2016, a CEMIG já havia investido mais de 10 milhões de reais em projetos de P&D para abordar o problema decorrente da invasão ocasionada por *L. fortunei* (Silva *et al.*, 2016).

Esta capacidade de obstruir a passagem de água nas tubulações está relacionada com a produção de fios de bisso, que são filamentos secretados por glândulas localizadas no pé de *L. fortunei*. A adesão entre o bisso e o substrato se dá em escala nanométrica, por interações do tipo capilaridade e forças de van der Waals (Meyers, 2008; Andrade *et al.*, 2015), permitindo que o mexilhão-dourado se fixe em praticamente qualquer substrato sólido, como pedras, troncos e conchas de outros moluscos a cascos de barco, redes de pesca, placas de vidro e teflon (Faria et al., 2006). A formação dos filamentos do bisso mostra-se extremamente organizada, sincronizada e complexa, envolvendo a presença de vesículas contendo colágeno na fase de cristal líquido, que parecem estar envolvidas na montagem desses filamentos (Renner-Rao et al., 2019).

No entanto, é difícil identificar as larvas de moluscos bivalves. Técnicas de identificação ópticas não funcionam com a maior parte dessas larvas, uma vez que os caracteres específicos da espécie surgem apenas nos estágios mais tardios do desenvolvimento ou estão visíveis apenas em um nível microscópico (Garland & Zimmer, 2002). Assim, a utilização de métodos moleculares vem sendo empregada e mostra-se como uma alternativa para corroborar com a identificação morfológica, tão precoce quanto possível, dessas larvas em amostras de água (Pie et al., 2006; Marescaux & Van Doninck, 2013; Pie et al., 2017; Oliveira Júnior et al., 2018).

No contexto dos problemas ambientais e das perdas econômicas decorrentes dos processos de bioincrustação e associados aos danos ocasionados pelas invasões biológicas, faz-se necessário o incentivo de estudos que integrem, corroborem e ampliem o conhecimento acerca desses processos. Nesse sentido, o CBEIH visa a integração indissociável entre universidades, centros de pesquisa, poderes público e privado e a sociedade, entes estes que, em conjunto, visem e se responsabilizem pela prevenção, controle ou erradicação dos organismos bioincrustantes e evitem a disseminação das espécies invasoras. Para tanto, a detecção de tais organismos, sejam eles micro e/ou macroincrustantes, é o primeiro passo para se determinar os meios de atuação e as estratégias mais eficientes, a fim de mitigar o problema. Dessa forma, o uso de ferramentas moleculares para a identificação destes organismos mostra-se promissor para o desenvolvimento eficiente de estratégias anti-incrustantes.

2. Objetivos

2.1. Objetivo Geral

Desenvolver e viabilizar o uso de ferramentas moleculares para a detecção de espécies bioincrustantes em hidrelétricas.

2.2. Objetivos Específicos

2.2.1. CAPÍTULO 1

I) Reconhecer bioassinaturas produzidas por ferrobactérias (FeOB) em sedimentos provenientes da Hidrelétrica de Irapé (UHE-Irapé);

I) Sequenciar o metagenoma de amostras de sedimento da caverna de percolagem e do paredão rochoso da Usina Hidrelétrica de Irapé (UHE-Irapé);

II) Identificar sequências do gene de RNAr (RNA ribossomal) 16S relacionadas à ferrobactérias, recuperadas destes metagenomas;

III) Construir um banco de dados contendo sequências do gene de RNAr 16S relacionadas à membros da família Gallionellaceae;

IV) Construir uma árvore filogenética contendo sequências relacionadas à Gallionellaceae de: metagenoma das amostras de sedimento da caverna de percolagem e do paredão rochoso; do sedimento e lama ferruginosa, presentes no sistema de resfriamento caracterizados em trabalho anterior do nosso grupo; e do banco de dados compilado; a fim de classificar membros da família Gallionellaceae em nível de gênero.

2.2.2. CAPÍTULO 2

I) Detectar molecularmente a presença do hidrozoário invasor *Cordylophora caspia* em amostras coletadas em hidrelétricas;

II) Caracterizar molecular e morfologicamente o hidrozoário invasor *C. caspia*;

III) Reportar novas ocorrências e as primeiras sequências genéticas de *C. caspia* em território brasileiro.

IV) Contribuir com a discussão filogenética sobre a classificação quanto à salinidade do ambiente que habitam e acerca do gênero *Cordylophora* abrigar espécies crípticas.

2.2.3. CAPÍTULO 3

I) Compilar e redigir uma revisão que discorra sobre os aspectos moleculares envolvendo a biologia e ecologia do bivalve invasor *Limnoperna fortunei*;

II) Redigir um artigo de opinião que ressalte a importância de envolver múltiplas esferas públicas e privadas para lidar com a invasão de *L. fortunei*;

III) Padronizar, laboratorialmente, a técnica de PCR-LAMP para detecção de *L. fortunei*.

3. Capítulo 1

Journal of Industrial Microbiology and Biotechnology

Diversity and distribution of iron-oxidising bacteria belonging to Gallionellaceae in different sites of a hydroelectric power plant

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Abstract:	Iron (Fe) is the fourth most abundant element on the planet and iron oxidising bacteria (FeOB) play an important role in the biogeochemical cycle of this metal in nature. FeOB stands out as Fe oxidizers in microaerophilic environments and new members of this group have been increasingly discussed in literature, even though their isolation can be challenging in some cases. Among these bacteria is the Gallionellaceae family, mainly composed of neutrophilic FeOB, highlighting Gallionella ferruginea , and nitrite-oxidizers genera. In a previous study by our group, in which we accessed the metagenome of microbial slime of the cooling system from the Irapé hydroelectric power plant (UHE-Irapé), Gallionellaceae-related sequences represented 5% of the total bacteria. Thus, in this study, we constructed a phylogenetic tree based on this family, in order to search for shared and unique Gallionellaceae signatures in a deep phylogenetic level affiliation and correlated them with geomorphologic characteristics. Gallionella and Ferrigenium were ubiquitous in the UHE-Irapé which reflected their

	ability to adapt to various locations in the power plant. The cave was considered a hotspot for neutrophilic FeOB since it harboured the most Gallionellaceae diversity, including Gallionella , Ferriphaselus, Sideroxydans and Candidatus Nitrotoga. Microscopic biosignatures were detected only in the CS1 sample, which presented abundance of the stalk-forming Ferriphaselus and of the sheath-forming Crenothrix . Further studies are required to provide more detailed insights on Gallionellaceae distribution and diversity patterns in hydroelectric power plants, particularly its biotechnological potential in this industry.
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Diversity and distribution of iron-oxidising bacteria belonging to Gallionellaceae in different sites of a hydroelectric power plant

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Abstract

Iron (Fe) is the fourth most abundant element on the planet and iron oxidising bacteria (FeOB) play an important role in the biogeochemical cycle of this metal in nature. FeOB stands out as Fe oxidizers in microaerophilic environments and new members of this group have been increasingly discussed in literature, even though their isolation can be challenging in some cases. Among these bacteria is the Gallionellaceae family, mainly composed of neutrophilic FeOB, highlighting *Gallionella ferruginea*, and nitrite-oxidizers genera. In a previous study by our group, in which we accessed the metagenome of microbial slime of the cooling system from the Irapé hydroelectric power plant (UHE-Irapé), Gallionellaceae-related sequences represented 5% of the total bacteria. Thus, in this study, we constructed a phylogenetic tree based on this family, in order to search for shared and unique Gallionellaceae signatures in a deep phylogenetic level affiliation and correlated them with geomorphologic characteristics. *Gallionella* and *Ferrigenium* were ubiquitous in the UHE-Irapé which reflected their ability to adapt to various locations in the power plant. The cave was considered a hotspot for neutrophilic FeOB since it harboured the most Gallionellaceae diversity, including *Gallionella*, *Ferriphaselus*, *Sideroxydans* and *Candidatus* Nitrotoga. Microscopic biosignatures were detected only in the CS1 sample, which presented abundance of the stalk-forming *Ferriphaselus* and of the sheath-forming *Crenothrix*. Further studies are required to provide more detailed insights on Gallionellaceae distribution and diversity patterns in hydroelectric power plants, particularly its biotechnological potential in this industry.

Key words: Gallionellaceae, iron-oxidising bacteria, *Gallionella*, biosignatures, hydroelectric power plant

1. Introduction

Lithotrophic iron-oxidizing bacteria (FeOB) are major players in the iron (Fe)

biogeochemical cycle, with the ability to catalyse the dissimilatory oxidation of ferrous to ferric iron (Emerson et al., 2013). Currently, FeOB are subdivided into four physiological groups: (1) acidophilic, aerobic FeOB; (2) neutrophilic, aerobic FeOB; (3) neutrophilic, nitrate-dependent anaerobic FeOB; and (4) anaerobic, photosynthetic FeOB (Khalifa et al., 2018). Acidophilic FeOB, such as *Acidithiobacillus ferrooxidans*, have been exhaustively studied due to their potential in the remediation of acidic mines (Johnson & Hallberg, 2005; Wendt-Potthoff et al., 2010), whereas the role of neutral FeOB has only been documented in the last 20 years (Emerson & Floyd, 2005; Emerson et al., 2010). The main reasons for these differences were (i) the rapid abiotic oxidation of Fe in neutral and oxygenated habitats, that might not be competitive with biotic condition, resulting in fewer studies of this group (Hedrich et al., 2011); and (ii) the challenge of isolating pure cultures, as they live in an opposing gradient of Fe (II) and oxygen (Emerson et al., 2010). However, it has since been proven that the oxidation process performed by microaerophilic bacteria can compete with the abiotic Fe (II), reaching up to 60% of the total Fe oxidation in neutral habitats (Emerson & Moyer, 2002). Furthermore, molecular analysis has proven to be useful in the identification of new species in this group, as well as in the comparative studies, emphasising their ecology and genomic repertoire (Wang et al., 2009; Emerson et al., 2013; Reis et al., 2014; Bethencourt & Kunze, 2020). Stalk, dread and sheath morphologies can also confidently be associated with FeOB metabolism and have been described as extracellular biosignatures (Chan et al., 2011; Kato et al., 2015).

A part of this group, Gallionellaceae-related FeOB are abundant in freshwater environments and are often retrieved from redox transition boundaries, where they benefit from both high Fe concentrations and microaerophilic conditions (Wang et al., 2009; Emerson et al., 2010; Hedrich et al., 2011). This clade contains the well-studied and broadly distributed *Gallionella ferruginea*, which has been found in various habitats such as soils, freshwater springs and deep sea hydrothermal fields (Halbach et al., 2001; Reis et al., 2014). *Gallionella ferruginea* and *Ferriphaselus*, another Gallionellaceae FeOB isolated from groundwater seep (Kato et al.,

2014), are both stalk-forming bacteria, a characteristic that prevents them from encrusting with iron oxides (Hallbeck & Pedersen, 1995; Kato et al., 2014). *Sideroxydans* has also been isolated in groundwater, able to oxidise iron in humic acid-enriched cultures, despite being poorly represented in mining areas (Emerson & Moyer, 1997; Reis et al., 2014; Hädrich et al., 2019). *Ferrigenium kumadai*, recently identified and classified as Gallionellaceae-related FeOB, was isolated from paddy field soil (Khalifa et al., 2018). The Gallionellaceae family also harbours members that are not FeOB such as, *Candidatus* Nitrotoga arctica and *Candidatus* Nitrotoga fabula, which are nitrite-oxidizers (Alawi et al., 2007; Kitzinger et al., 2018; Zheng et al., 2020).

In a previous metagenomics study performed by our group (Reis et al., 2021), we aimed to characterise microbial communities of the cooling internal system of Irapé hydroelectric power plant (Minas Gerais, Brazil), especially particular taxonomic signatures of biofilm formation in heat exchangers, associated with biofouling formation. As the region in the previous study is iron- and sulphur-rich, it can be considered a hotspot for FeOB survey. Bethencourt & Kunze (2020) revealed through genome sequencing, 11 candidate genera recovered from two redox transition zones. The genes suggested that dissimilatory sulphur and nitrogen pathways are part of the Gallionellaceae's energetic metabolism, together with iron oxidation. Moreover, among the obtained results, the authors unveiled Gallionellaceae OTUs abundance of 5% from the total of bacteria being 99% unclassified at genus level, which reflected the high number of uncultured Gallionellaceae-related members (Reis et al., 2021). Thus, in the current study a phylogenetic tree was proposed in order to complement the metagenomics data on FeOB members. Finally, the authors looked for morphological FeOB biosignatures in the samples, using scanning electron microscopy (SEM), to find other FeOB traces in this power plant.

2. Material and Methods

2.1 Study area

The Irapé hydroelectric power plant is located in the north of Minas Gerais state, Brazil

(UHE-Irapé; 16°44'15"S, 42°34'30"W) a region rich in iron and sulphur (Figure S1). UHE-Irapé belongs to Companhia Energética de Minas Gerais (CEMIG) and is considered the tallest dam in the country, 208 m high with a flooded area of 137.16 km^2 located in the course of the Jequitinhonha River, generating 399 MW. Four sites were sampled, two of them located outside of the power plant: rock sediment (RS) and artificial cave sediment (CS) (reads from both locations were deposited in the Sequence Read Archive (SRA) - NCBI under submission number PRJNA643245); and other two were taken from part of the cooling water system, previously characterised by metagenomics (submission number on SRA - NCBI: PRJNA628802; Reis et al., 2021): filter sediment (FS) and heat exchanger microfouling (HEM). Two sampling campaigns were performed first in September 2015 (campaign 1) and again in February 2016 (campaign 2).

We performed the total DNA isolation extraction followed by the sequencing of the V4 variable region of 16S rRNA gene from RS1, RS2, CS1 and CS2 together with the other power plant samples described in Reis et al. (2021). We used the E.Z.N.A.® Soil DNA Kit (OMEGA Bio-tek, Norcross, GA, USA) for the Total DNA isolation and the MiSeq platform (Illumina, Inc., San Diego, CA, USA) for the sequencing. Finally, all eight samples were geomorphologically evaluated by scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDS).

2.2 Bioinformatics analysis

2.2.1 16S amplicon reconstruction from Next-Generation Sequencing (NGS) data

Quality evaluation was performed on the NGS sequence data from the metagenomics samples using MultiQC (v1.10.1) (Ewels et al., 2016). BBDuk (Bushnell et al., 2014) was used to trim low quality edges and adapter sequences. An amplicon sequence variant table was generated for the 16S variants on the metagenomics samples using the DADA2 package (v1.20) (Callahan et al., 2016) from the Bioconductor repository for the R platform (v4.1.0). Amplicons

were taxonomically classified by the DADA2 package using the Silva reference database (v138.1) (Quast et al., 2013). Amplicons classified as pertaining to the Gallionellaceae family were exported for phylogenetic tree construction.

2.2.2 Phylogenetic tree construction

Reference rRNA 16S gene sequences of 17 members of the Gallionellaceae family were obtained from the Silva Species Assignment training dataset maintained by the DADA2 package, alongside 1 reference sequence of *Methylobacillus pratensis*, which served as an outside group. A 16S sequence of *Ferrigenium kumadai* An22 was obtained from the EBI/ENA database (Entry LC065124). The evolutionary history of the 13 metagenomics sequences alongside the 19 reference sequences was inferred using the UPGMA method (Sneath & Sokal, 1973). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and the rate variation among sites was modelled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1,544 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

2.3 Geomorphological analysis of sediments and microfouling samples from UHE-Irapé

2.3.1 Scanning Electron Microscopy (SEM)

Sediments and microfouling were fixed in glutaraldehyde 2.5% and dehydrated in ethanol series ranging from 70% to 100%. Some of the CS samples were treated with bleach (NaClO 2%-5%) and others in a muffle furnace at 800 °C. Aluminium stubs were mounted with 10-20 µL of all samples (RS, CS, FS and HEM) in ethanol 100% on carbon adhesive tape. Further, samples were coated with a blend of gold-palladium (Quorum coater sc7620) in an argon atmosphere for 30-60 seconds. SEM sessions were performed in a high vacuum atmosphere with LM-VEGA3-TESCAN SEM. Images and biomineral measurements were acquired and made

after settling adequate working distance, beam intensities, magnification and astigmatism, as well as column and centralization of the electronic cannon, when it was necessary. The software used for those was the Vega3 Control version (version 4.2.4.0).

2.3.2 Energy Dispersive X-ray Spectroscopy (EDS)

The biomineral chemical analysis was characterised by EDS, detected with PentaFET® Precision, x-act and processed on Aztec 2.0 software (Oxford Instruments). The analyses were performed using the Map and Point & ID modes. Most of the analyses were performed with 20kV, BI = 16, as they generated a greater signal in the EDS and a dead time of around 15%-20%. The elements were pre-defined in the periodic table available in the software which also completed the Auto ID. Elements were first identified by Auto ID and then fixed for further analyses.

3. Results and discussion

FeOB oxidises iron as a result of their metabolism, which means that the members of this diverse group are able to use the electrons captured from this oxidation as their sole source of energy for growth (Emerson, 2018). In attempt to characterise the distribution and diversity of FeOB, mainly Gallionellaceae-related members recovered from sediments and microfouling samples in UHE-Irapé, our analysis searched for shared and unique Gallionellaceae signatures in a deep phylogenetic level affiliation and correlated them with geomorphologic characteristics in those samples.

Figure 1 and table S1 represent the FeOB described in the literature until now, highlighting those previously found in the metagenomes of different samples from UHE-Irapé, as well as their abundance in each sample. In RS2 prevailed *Acidithiobacillus* (aerobic), *Ferritrophicum* (microaerophilic) and *Leptospirillum* (aerobic), which are acidophilic bacteria able to oxidise iron containing sulphide minerals, such as pyrite (Harneit et al., 2006; Weiss et

al., 2007; Johnson, 2015). The abundance of these species are expected since the principal compounds that characterise soils with low pH are those containing iron and sulphur, commonly found in the sampling region. The Gallionellaceae genus *Ferriphaselus* predominated in CS1 and *Acidiferrobacter* in CS2. *Hyphomicrobium* was abundant in CS1, CS2 and FS1 and passively oxidises Fe^{2+} and precipitates FeO(OH) (Konhauser, 1998), being also associated with Mn oxidation (Stein et al., 2001). *Meiothermus* (mesophilic) and *Thermomonas* (thermophilic) genera harbour iron-oxidising species (Kozubal et al., 2008; Hedrich et al., 2011), and were significantly found in HEM2_15 being considered putative pioneering colonisers in microfouling of the heat exchanger (Reis et al., 2021). *Sediminibacterium* was also abundant in HEM2_15 and it was revealed by Wang et al. (2012) as a corrosion-inducing bacterium.

Indeed, the construction of a phylogenetic tree to predict taxonomic affiliation from clustered sequences is an approach already recommended by other studies as an efficient tool to complement the metagenomics findings (McDonald et al., 2012; Yilmaz et al., 2014; Edgar, 2018). The five described genera affiliated to Gallionellaceae are represented in the phylogenetic tree: *Gallionella*, *Ferriphaselus*, *Syderoxydans, Ferrigenium, Candidatus* Nitrotoga, being four of them FeOB and one a nitrite-oxidising bacterium (*Ca.* Nitrotoga) (Figs. 1 and 2).

According to the phylogenetic tree, *Ferrigenium* was the most abundant and ubiquitous genus in the power plant, being found in CS1, FS1, FS2 and HEM2_15 and exclusive in FS1, FS2 and HEM2_15 samples, while *Sideroxydans*, *Ferriphaselus* and *Ca.* Nitrotoga were found in CS1 (Fig. 2 and Table S1). Otherwise, Gallionellaceae sequences recovered from metagenomes analyses identified *Gallionella* as the most ubiquitous genus, being found in CS1, FS2, HEM1_30 and HEM2_15. Possibly the ability of *Gallionella* to deal successfully in environments with the presence of potentially toxic metals and metalloids, due to its resistance genes repertoire (Emerson et al., 2013; Reis et al., 2014), favoured its ubiquity in the power plant. However, *Ferrigenium* was characterised by not utilising hydrogen, thiosulfate, sulphide, nitrite, Mn(II), glucose, acetate, pyruvate or citrate as an energy source (Khalifa et al., 2018), presenting

a lifestyle more restrictive than *Gallionella*, despite being also ubiquitous in this environment.

Microscopic analysis detected the presence of stalks and sheaths, which are biosignatures of Betaproteobacteria occurrence, only in the sediments from CS1 (Figure 3). As reported by Chan et al. (2009 and 2011), stalks are carboxyl-rich polysaccharides structures that may prevent cell encrustation with iron oxides. Otherwise, sheaths are described as an empty tube, initially made of a complex of a cysteine-rich peptide and a polysaccharide composed of uronic acids and galactosamine (Emerson & Ghiorse, 1993), which is followed by a later deposition of iron and manganese previously oxidised by the cells (Mulder & Deinema, 1981). This empty tube surrounds a chain of cells enabling these bacteria to attach to solid surfaces where it can not be consumed by protozoa or attacked by predators such as *Bdellovibrio bacteriovorus*, it can also work as a physical barrier against other microorganisms (Mulder & Deinema, 1981). These biosignatures may also contribute to the maintenance and structuring of bacterial biofilms, since as reported by Kato et al. (2015), stalks can work like an anchor to attach to solid structures preventing the cell from being disconnected from it. Surprisingly, although stalk-forming bacteria (Weiss et al., 2007; Kato et al., 2014) were also detected in HEM (*Gallionella* and *Ferriphaselus*) (Fig 3, A-C), stalks were not found in the heat exchanger samples, where microbial slime (biofilm and sediment) was formed (Fig. 3J) (Reis et al., 2021).

Sheath-forming bacteria are often associated with *Leptothrix* (Chan et al., 2016) despite this not being the unique genus that produces it. *Sphaerotilus*, *Crenothrix polyspora*, *Calothrix* and *Lieskeella bifida* have also been described as iron-oxidising sheath-forming bacteria (Ghiorse, 1984; Kappler et al., 2015). From these bacteria, only *Crenothrix* was recovered by our previous metagenomic survey (Reis et al., 2021). Indeed, it is not possible to identify the producer of the sheaths based on the microscopy (Fig 3., B, D, E-I). However, in spite of these two hypotheses were formed: (1) the sheaths were produced by *Crenothrix* recovered in CS1; (2) since these structures are recognized as microfossils due to their persistence in the environment and thermostability (600-1100 ºC) (Ellis, 2003; Chan et al., 2011; Hashimoto et al., 2012), they

could be produced by other sheath-forming bacteria that are no longer present in CS, such as *Leptothrix*.

Otherwise, *Ca.* Nitrotoga is related to nitrite oxidation, an important process for removing nitrogen from wastewater (Klotz & Stein, 2011). *Candidatus* Nitrotoga fabula, the first genus isolated from activated sludge, previously considered an environment entirely dominated by *Nitrospira*, develops at higher temperatures (20 °C; optimum, 24 to 28°C) and adapts to low-oxygen or potentially anoxic conditions when compared to *Ca.* Nitrotoga artica, which are cold-adapted nitrite oxidizers isolated from permafrost and that grow optimally at temperatures as low as 10 °C (Alawi et al., 2007; Lücker et al., 2015; Boddicker & Mosier, 2018; Kitzinger et al., 2018). Thus, *Ca.* Nitrotoga supports psychrophilic and mesophilic conditions and is widespread in engineered and natural ecosystems (Kitzinger et al., 2018; Zheng et al., 2020). Besides, it has been demonstrated that *Sideroxydans* is able to occupy niches depleted in N, due to its three clusters of *nif* genes (Emerson et al., 2013; Reis et al., 2014). Thus, the presence of both bacteria in CS1 could be related to the metabolism of nitrogen in this environment, with the first removing nitrogen by nitrite oxidation and the second benefiting itself from the nitrogen lacking environment.

UHE-Irapé is located in an iron- and sulphur-rich region, probably favouring the abundance of bacteria with a key role in these biogeochemical cycles. In particular, CS and RS samples presented large amounts of sulphur (Fig. S1). Kato et al. (2015) unveiled genes related to sulphur oxidation in strains of *Ferriphaselus*, reinforcing the massive occurrence of this genus in the cave.

In conclusion, our data revealed the distribution and diversity of Gallionellaceae-related sequences, previously recovered from a metagenomic survey, and also analysed herein, by a phylogenetic tree construction. We observed differences between the Gallionellaceae classification from both analyses, allowing us to deep in the discussion about its distribution. After the phylogenetic analysis we detected a Gallionellaceae diversity more complex than those

presented by Reis et al. (2021). The ubiquity of *Gallionella* and *Ferrigenium* reinforced their plasticity to adapt to different locations of the power plant. Besides, *Gallionella*, *Ferriphaselus, Sideroxydans* and *Ca*. Nitrotoga were also found in the cave, indicating that this sample harbours more Gallionellaceae diversity, being a hotspot for neutrophilic FeOB.

Moreover, microscopic biosignatures such as stalks and sheaths were found only in CS1. *Ferriphaselus* possibly being responsible for producing the stalks, and *Crenothrix* likely having produced the sheaths. This is the most likely explanation as evidence of both of these bacteria were found to have previously inhabited the cave. Further studies are required to provide more insights on Gallionellaceae distribution and diversity patterns in hydroelectric power plants.

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FIGURES

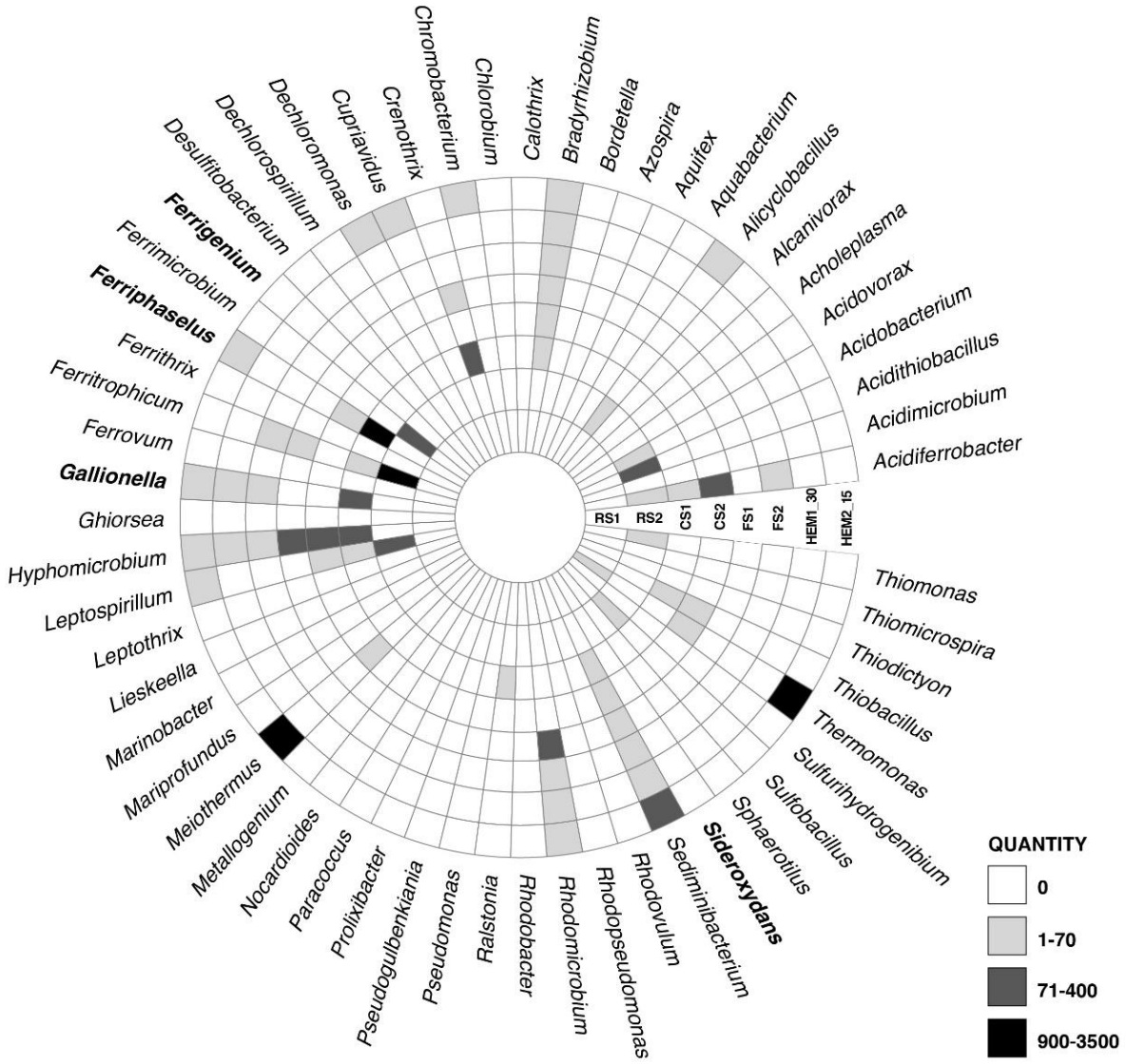


Figure 1. Abundance of iron oxidizing bacteria (FeOB) distributed at Irapé hydroelectric power plant (UHE-Irapé). The color intensity represents the quantity of recovered sequences of FeOB genera obtained from metagenomes of sampling sites. **RS:** rock sediment, **CS:** cave sediment, **FS:** filter sediment, **HEM:** heat exchanger microfouling. The numbers 1 and 2 after acronyms represent the sampling campaigns.

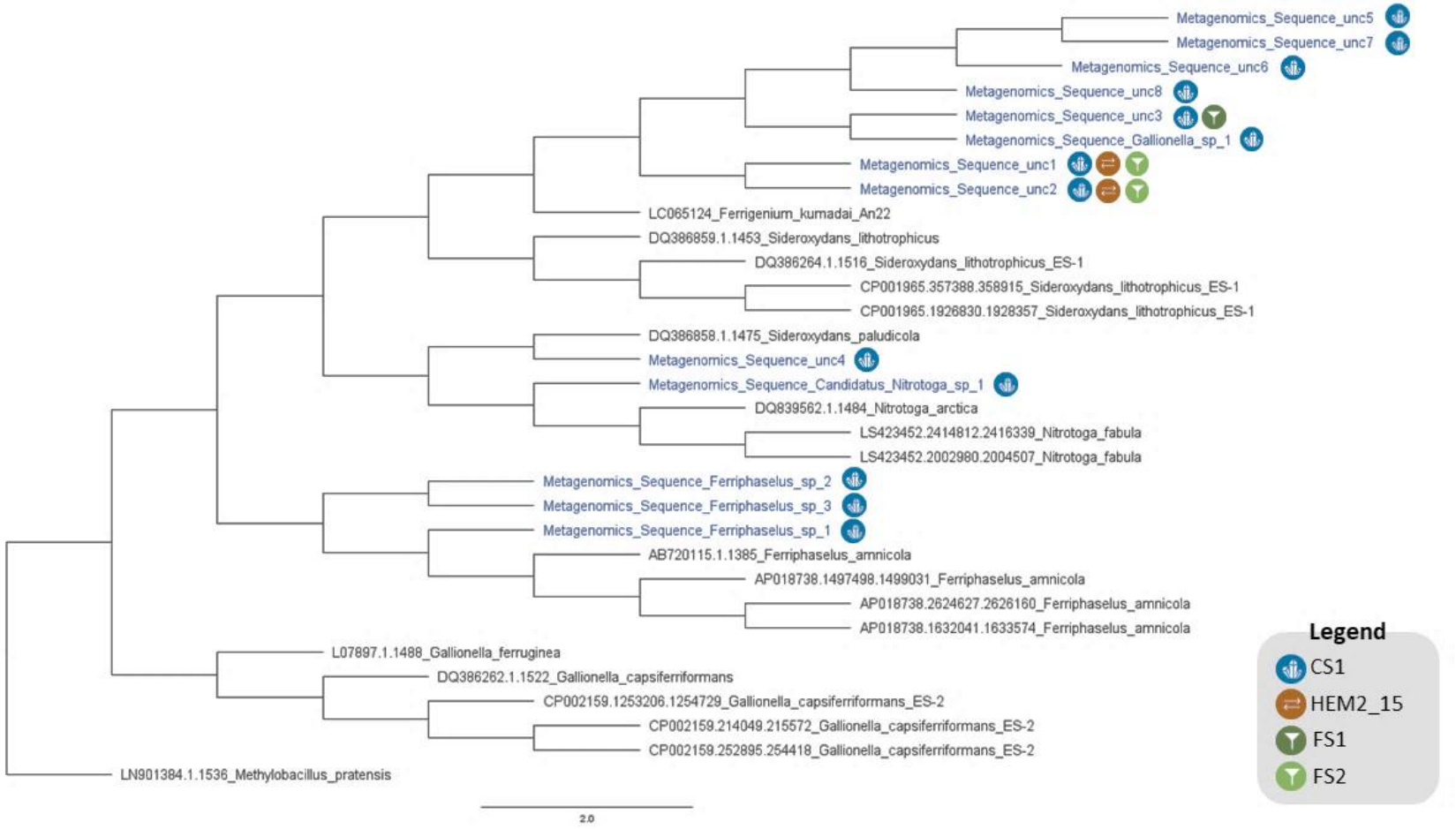
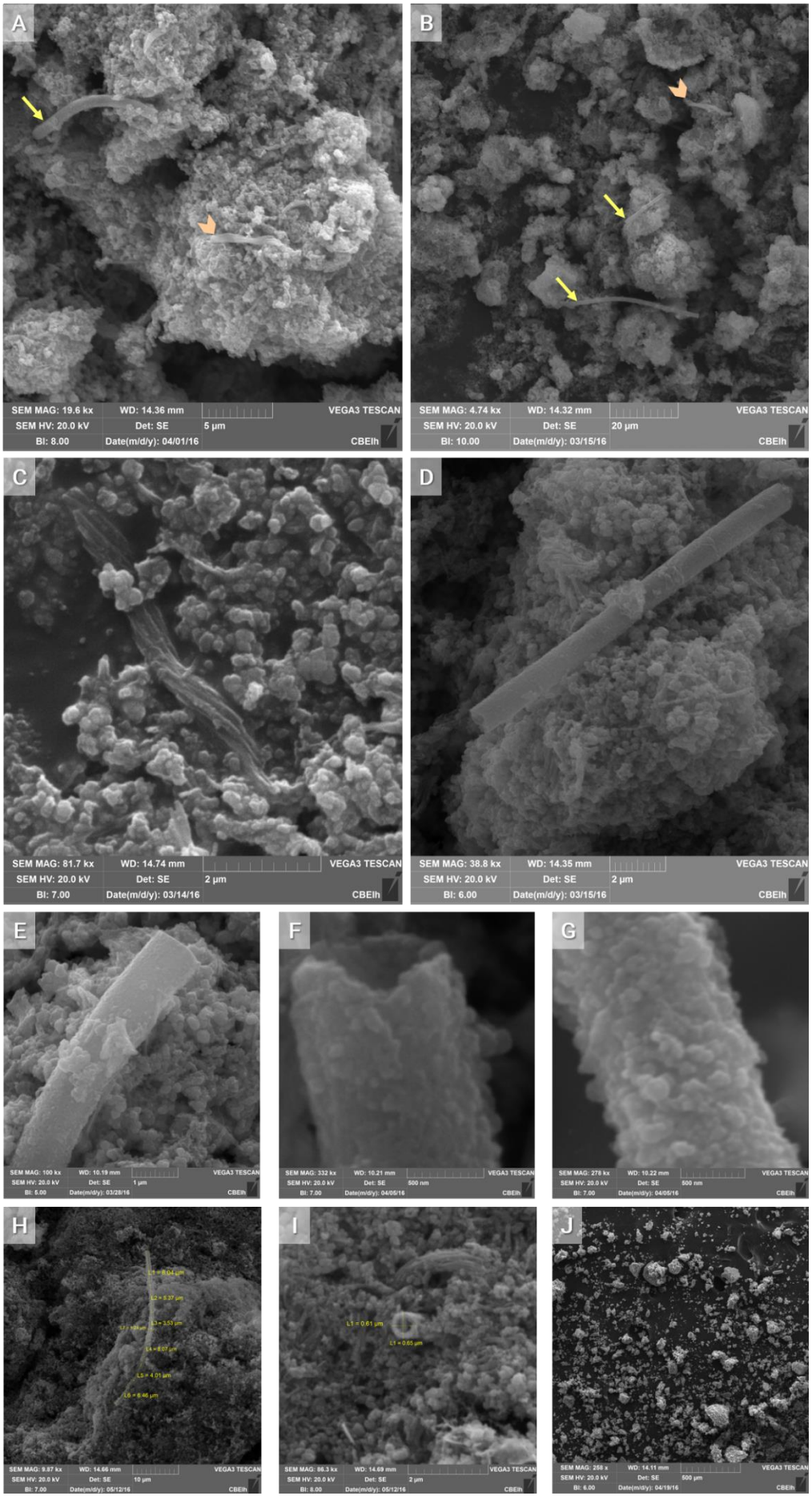


Figure 2. Phylogenetic tree showing recovered sequences at Irapé hydroelectric power plant (UHE-Irapé) with Gallionellaceae-related genera. RS: rock sediment, **CS:** cave sediment, **FS:** filter sediment, **HEM:** heat exchanger microfouling. The numbers 1 and 2 after acronyms represent the sampling campaigns.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WD: 14.36 mm
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WD: 14.74 mm
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Figure 3. ***Scanning Electron Microscopy*** **(SEM) of cave sediment (CS). A-B.** Sheaths (yellow arrows) and stalks (pink arrowhead) found in CS. **C.** Zoom highlighting stalk in the center of the micrograph. **D-E.** Zoom highlighting sheaths on micrographs. **F-G.** SEM of sheaths impregnated with sediment. **H.** Sheaths extension measurement in SEM. **I.** Sheaths diameter measurements. **J.** Sediment of heat exchanger without stalks and sheaths.

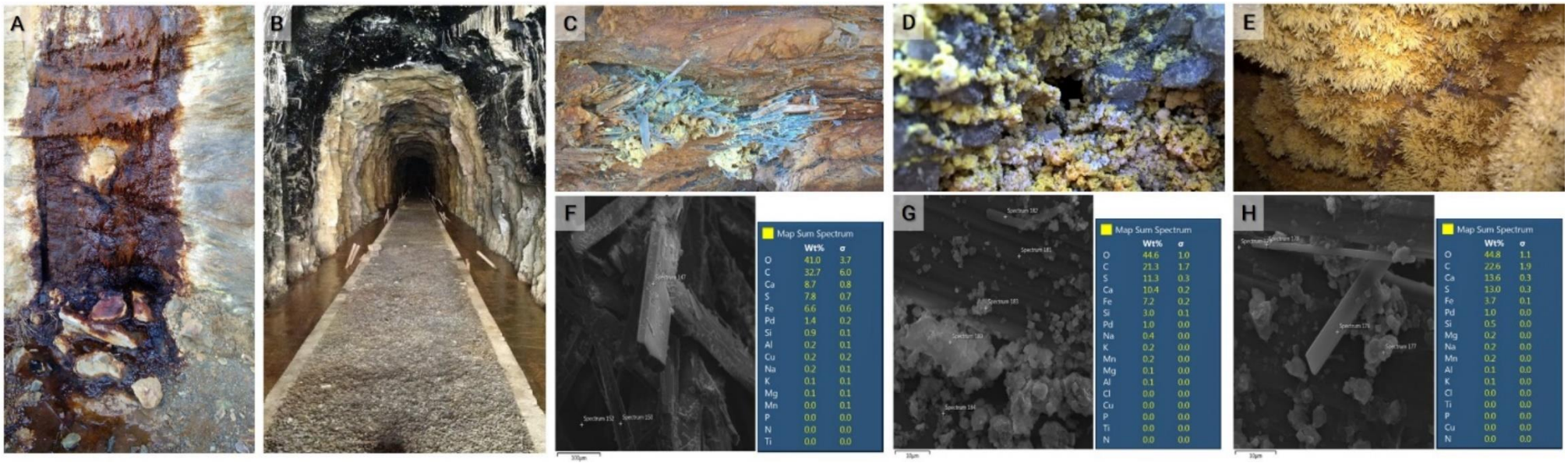

Figure S1. Geomorphological evaluation of UHE-Irapé sediments by scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDS). A. Photograph of sulfur-rich rock sediment. **B.** Photograph of inner cave wall with crystals of sulfur. **C-D.** Zoom on rock massif, evidencing large amounts of sulfur linked to other elements. **E.** Zoom on the inner wall of the cave covered by sulfur crystals. **F.** SEM of the sulfur-rich sediments of the cave inner wall. EDS analysis showing the amount of sulfur and other elements distributed in the analyzed micrograph. **G-H.** Zoom highlighting the sediments of the cave. EDS analysis shows the amount of sulfur and other elements distributed in the analyzed micrograph. The high values for the carbon element (C) due to the sample preparation, since the sediments are fixed on a carbon adhesive type before being conducted to the SEM and EDS evaluations. Chemical elements (in order of appearance): O: oxygen, C: carbon, Ca: calcium, S: sulfur, Fe: iron, Pd: palladium, Si: silicon, Al: aluminum, Cu: copper, Na: sodium, K : Potassium, Mg: magnesium, Mn: manganese, P: phosphorus, N: nitrogen, Ti: titanium.

TABLES

Table S1. and FeOB genera described in the literature and abundance of metagenomics sequences FeOB-related genera per sampling site. The genera listed have at least one species referenced as FeOB. **syn:** synonym, **str:** strain, **RS:** rock sediment, **CS:** cave sediment, **FS:** filter sediment, **HEM:** heat exchanger microfouling. The numbers 1 and 2 after acronyms represent the sampling campaigns.

Genera	Number of Sequences per Sampling Site								FeOB species	References
	RS1	RS2	CS1	CS2	FS1	FS2	HEM1_ 30	HEM2_ 15		
Acidiferrobacter	0	1	48	219	0	1	0	0	*Acidiferrobacter thiooxydans*	Hallberg et al., 2011
Acidimicrobium	0	0	0	0	0	0	0	0	*Acidimicrobium ferrooxidans*	Clark & Norris, 1996
Acidithiobacillus	0	202	0	0	0	0	0	0	*Acidithiobacillus ferrianus*	Norris et al., 2020
									Acidithiobacillus ferridurans	Hedrich & Johnson, 2013
									Acidithiobacillus ferriphilus	Falagan & Johnson, 2016
									Acidithiobacillus ferrivorans	Hallberg et al., 2010
									Acidithiobacillus ferrooxidans (syn. *Thiobacillus ferrooxidans*)	Temple & Colmer, 1951
Acidobacterium	0	23	0	0	0	0	0	0	*Acidobacterium capsulatum*	Weber et al., 2006
Acidovorax	0	0	0	0	0	0	0	0	*Acidovorax ebreus*	Byrne-Bailey et al., 2010
									Acidovorax sp. str. BoFeN1	Pantke et al., 2012; Klueglein & Kappler, 2013
									Acidovorax sp. str. BrG1	Weber et al., 2006
Acholeplasma	0	0	0	0	0	0	0	0	*Acholeplasma laidlawii* (syn. *Sapromyces laidlawi*)	Reinards et al., 1981
Alcanivorax	0	0	0	0	0	0	0	0	*Alcanivorax sp. str.* 3B.1	Makita, 2018
Alicyclobacillus	0	16	0	0	0	0	0	12	*Alicyclobacillus aeris*	Guo et al., 2009
									Alicyclobacillus disulfidooxidans (syn. *Sulfobacillus disulfidooxidans*)	Dufresne et al., 1996
									Alicyclobacillus ferrooxydans	Jiang et al., 2008

									Alicyclobacillus montanus	López et al., 2018
									Alicyclobacillus tolerans (syn. *Sulfobacillus thermosulfidooxidans*)	Karavaiko et al., 2005
Aquabacterium	0	0	0	0	0	0	0	0	*Aquabacterium* str. BrG2	Weber et al., 2006
Aquifex	0	0	0	0	0	0	0	0	*Aquifex aeolicus*	Pandelia et al., 2011
Azospira	0	0	0	0	0	0	0	0	*Azospira suillum* (syn. *Dechlorosoma suillum*)	Weber et al., 2006
Bordetella	0	0	0	0	0	0	0	0	*Bordetella sp.* strain FB-8	Harris et al., 2021
Bradyrhizobium	0	0	34	33	57	11	48	41	*Bradyrhizobium japonicum* str. *22*	Benzine et al., 2013
									Bradyrhizobium japonicum str. is5	
									Bradyrhizobium japonicum str. in8p8	
Calothrix	0	0	0	0	0	0	0	0	*Calothrix pulvinata*	Brayner et al., 2009
Chlorobium	0	0	0	0	0	0	0	0	*Candidatus* Chlorobium masyuteum	Lambrecht et al., 2021
									Chlorobium ferrooxidans	Weber et al., 2006
Chromobacterium	0	0	0	0	0	0	0	2	*Chromobacterium* str. 2002	Weber et al., 2006
Crenothrix	0	0	89	0	2	0	0	0	*Crenothrix polyspora*	Ghiorse, 1984
Cupriavidus	0	0	0	0	0	0	0	2	*Cupriavidus necator* str. A5-1	Benzine et al., 2013
Dechloromonas	0	0	0	0	0	0	0	6	*Dechloromonas agitata* str. is5	Benzine et al., 2013
Dechlorospirillum	0	0	0	0	0	0	0	0	*Dechlorospirillum sp.* str. M1	Picardal et al., 2011
Desulfitobacterium	0	0	0	0	0	0	0	0	*Desulfitobacterium frappieri* str. G2	Shelobolina et al., 2003
Ferrigenium	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	*Ferrigenium kumadai*	Khalifa et al., 2018
Ferrimicrobium	0	76	0	0	0	0	0	0	*Ferrimicrobium acidiphilum*	Johnson et al., 2009
Ferriphaselus	**0**	**0**	**3206**	**27**	**0**	**0**	**0**	**7**	*Ferriphaselus amnicola*	Kato et al., 2014
Ferrithrix	0	20	0	0	0	0	0	0	*Ferrithrix thermotolerans*	Johnson et al., 2009
Ferritrophicum	0	958	1	0	2	2	0	0	*Ferritrophicum radicicola*	Weiss et al., 2007
Ferrovum	0	0	0	0	0	0	0	0	*Ferrovum myxofaciens*	Johnson et al., 2014
Gallionella	**0**	**0**	**97**	**0**	**0**	**12**	**1**	**36**	*Candidatus* Gallionella acididurans	Kadnikov et al., 2016

									Gallionella capsiferriformans str. ES-2	Emerson et al., 2013
									Gallionella ferruginea	Ehrenberg, 1836; Weber et al., 2006; Emerson, 2018
Ghiorsea	0	0	0	0	0	0	0	0	*Ghiorsea bivora*	Mori et al., 2017
Hyphomicrobium	0	0	290	226	341	48	10	64	*Hyphomicrobium sp.*	Ionescu et al., 2015
Leptospirillum	0	296	6	3	0	0	0	1	*Leptospirillum ferriphilum*	Coram & Rawlings, 2002; Penev & Karamanev, 2010
									Leptospirillum ferrodiazotrophum	Tyson et al., 2005; Goltsman et al., 2009
									Leptospirillum ferrooxidans	Hippe, 2000
									Leptospirillum rubarum	Goltsman et al., 2009
									Leptospirillum thermoferrooxidans	Golovacheva et al., 1992; Hippe, 2000
Leptothrix	0	0	0	0	0	0	0	0	*Leptothrix cholodnii*	Spring et al., 1996; Vollrath et al., 2013
									Leptothrix discophora	Corstjens et al., 1992; Spring et al., 1996; Weber et al., 2006
									Leptothrix ginsengisoli	Baskar et al., 2012
									Leptothrix lopholea	Mulder & Van Veen, 1963
									Leptothrix mobilis	Spring et al., 1996; Sung et al., 2011
									Leptothrix ochracea	Hashimoto et al., 2007; Fleming et al., 2018
Lieskeella	0	0	0	0	0	0	0	0	*Lieskeella bifida*	Ghiorse & Ehrlich, 1993
Marinobacter	0	0	0	0	0	0	0	0	*Marinobacter aquaeolei*	Weber et al., 2006; Singer, Webb, et al., 2011
									Marinobacter hydrocarbonoclasticus	Penas et al., 2019
									Marinobacter salsuginis	Khan et al., 2020
									Marinobacter subterrani	Bonis & Gralnick, 2015
Mariprofundus	0	0	0	0	0	0	0	0	*Mariprofundus aestuarium*	Chiu et al., 2017
									Mariprofundus erugo	Garrison et al., 2019
									Mariprofundus ferrinatatus	Chiu et al., 2017
									Mariprofundus ferrooxydans	Singer, Emerson, et al., 2011
									Mariprofundus micogutta	Makita et al., 2017

Meiothermus	0	0	0	7	0	0	0	3205	*Meiothermus sp.*	Selvarajan et al., 2018
Metallogenium	0	0	0	0	0	0	0	0	*Metallogenium sp.*	Walsh & Mitchell, 1972
Nocardioides	0	0	0	0	0	0	0	0	*Nocardioides sp. strain in31*	Benzine et al., 2013
Paracoccus	0	0	0	0	0	0	0	0	*Paracoccus denitrificans*	Park et al., 2018
									Paracoccus ferrooxidans	Kumaraswamy et al., 2006
									Paracoccus pantotrophus str. KS1	Price et al., 2018
Prolixibacter	0	0	0	0	0	0	0	0	*Prolixibacter bellariivorans*	Iino et al., 2015; Makita, 2018
									Prolixibacter denitrificans	Iino et al., 2015; Makita, 2018
Pseudogulbenkiania	0	0	0	0	0	0	0	0	*Pseudogulbenkiania ferrooxidans* str. 2002	Byrne-Bailey et al., 2012
									Pseudogulbenkiania sp. str. MAI-1	Kopf et al., 2013
Pseudomonas	0	0	0	0	0	0	0	0	*Pseudomonas stutzeri*	Weber et al., 2006
Ralstonia	0	0	1	0	0	0	0	0	*Ralstonia solanacearum*	Shelobolina et al., 2012
									Ralstonia sp.	Swanner et al., 2011
Rhodobacter	0	0	0	0	0	0	0	0	*Rhodobacter capsulatus* str. SB1003	Poulain & Newman, 2009
									Rhodobacter ferrooxidans str. SW2	Weber et al., 2006; Hegler et al., 2008; Saraiva et al., 2012
Rhodomicrobium	0	0	0	0	93	5	2	1	*Rhodomicrobium udaipurense* str. JA643	Conners et al., 2021
									Rhodomicrobium vannielii	Heising & Schink, 1998; Weber et al., 2006
Rhodopseudomonas	0	0	0	0	0	0	0	0	*Rhodopseudomonas palustris* Str. TIE-1	Jiao et al., 2005
Rhodovulum	0	0	0	0	0	0	0	0	*Rhodovulum iodosum*	Straub et al., 1999; Weber et al., 2006
									Rhodovulum rubiginosum	Straub et al., 1999; Weber et al., 2006
Sediminibacterium	0	0	12	5	23	46	5	297	*Sediminibacterium sp.*	Emerson, 2018; Wu et al., 2019
Sideroxydans	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	*Sideroxydans lithotrophicus* str. ES-1	Emerson et al., 2013
									Sideroxydans paludicola	Weiss et al., 2007
Sphaerotilus	0	0	0	0	0	0	0	0	*Sphaerotilus natans*	Stokes, 1954
Sulfobacillus	0	1	0	0	0	0	0	0	*Sulfobacillus harzensis*	Zhang et al., 2021

									Sulfobacillus acidophilus	Brock & Gustafson, 1976
									Sulfobacillus benefaciens	Johnson et al., 2008
									Sulfobacillus sibiricus str. N1	Dinarieva et al., 2015
									Sulfobacillus thermosulfidooxidans	Brock & Gustafson, 1976
									Sulfobacillus thermotolerans str. Kr1	Tat'yana et al., 2005
Sulfurihydrogenibium	0	0	0	0	0	0	0	0	*Sulfurihydrogenibium azorense*	Aguiar et al., 2004
									Sulfurihydrogenibium subterraneum	Nakagawa et al., 2005
Thermomonas	1	0	0	4	0	0	0	3438	*Thermomonas sp.* str. BrG3	Straub et al., 1996
Thiobacillus	0	0	65	19	0	0	0	0	*Thiobacillus denitrificans*	Beller et al., 2005
Thiodictyon	0	0	0	0	0	0	0	0	*Thiodictyon sp.* strain F4	Schädler et al., 2009
Thiomicrospira	0	0	0	0	0	0	0	0	*Thiomicrospira sp.* str. SC-1	Barco et al., 2017
Thiomonas	0	6	0	0	0	0	0	0	*Thiomonas ferrovorans* str. FB-6	Akob et al., 2020
									Thiomonas metallidurans str. FB-Cd	Akob et al., 2020

4. Capítulo 2

Biological Invasions

Cordylophora caspia (Pallas, 1771) (Cnidaria, Hydrozoa) in Brazil: a review of the current scenario with new records, evolutionary inferences, and perspectives

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Abstract:	The invasion of non-native species has been a chronic problem in reservoirs of Brazilian hydroelectric plants. Among these species recorded in Brazil, Cordylophora caspia stands out because it is often misidentified as macrophytes or completely overlooked, although there are reports stating that the colonies obstruct structures and pipes in industrial plants, generating high maintenance costs. In addition to modifying the ecological balance of invaded ecosystems, studies indicated that C. caspia can influence the occurrence of other invasive species, such as Limnoperna fortunei . In this study, we reported two new records for C. caspia in Brazil, the first occurrence in the states of Minas Gerais and Goiás. We also described the colonies in detail and reviewed the known geographic distribution of the species in Brazilian waters. The morphological identification was corroborated by obtaining the first Brazilian genetic sequences of the species, which were phylogenetically placed into a broad evolutionary inference using C. caspia COI sequences available from different regions of the world. The positioning of new sequences indicates that there is no clear

	pattern of ecophysiological specialization of main clades regarding salinity that would justify the validation of the nominal C. lacustris . High levels of genetic differentiation among Brazilian samples support the existence of multiple cryptic species within the genus Cordylophora . Finally, our ancestral state reconstruction analysis suggested a presumed freshwater origin for C. caspia . Advances in the study of additional Brazilian populations are important for understanding possible routes of invasion, economic impacts, and ecological interactions, including with other invasive species.
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Cordylophora caspia (Pallas, 1771) (Cnidaria, Hydrozoa) in Brazil: a review of the current scenario with new records, evolutionary inferences, and perspectives

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ABSTRACT

The invasion of non-native species has been a chronic problem in reservoirs of Brazilian hydroelectric plants. Among these species recorded in Brazil, *Cordylophora caspia* stands out because it is often misidentified as macrophytes or completely overlooked, although there are reports stating that the colonies obstruct structures and pipes in industrial plants, generating high maintenance costs. In addition to modifying the ecological balance of invaded ecosystems, studies indicated that *C. caspia* can influence the occurrence of other invasive species, such as *Limnoperna fortunei*. In this study, we reported two new records for *C. caspia* in Brazil, the first occurrence in the states of Minas Gerais and Goiás. We also described the colonies in detail and reviewed the known geographic distribution of the species in Brazilian waters. The morphological identification was corroborated by obtaining the first Brazilian genetic sequences of the species, which were phylogenetically placed into a broad evolutionary inference using *C. caspia COI* sequences available from different regions of the world. The positioning of new sequences indicates that there is no clear pattern of ecophysiological specialization of main clades regarding salinity that would justify the validation of the nominal *C. lacustris*. High levels of genetic differentiation among Brazilian samples support the existence of multiple cryptic species within the genus *Cordylophora*. Finally, our ancestral state reconstruction analysis suggested a presumed freshwater origin for *C. caspia*. Advances in the study of additional Brazilian populations are important for understanding possible routes of invasion, economic impacts, and ecological interactions, including with other invasive species.

Keywords: *Cnidaria, invasive species, hydroelectric power plant, phylogeny, populations, salinity.*

INTRODUCTION

Reservoirs are among the freshwater environments most favorable to colonization by non-native species (Vitule et al. 2012). In general, damming rivers change the availability of habitats and resources in addition to increasing physical disturbance and environmental variability (Havel et al. 2005a), transforming reservoirs into springboards for the introduction of non-native species in new hydrographic basins (Havel et al. 2005b). Countries that have a large number of reservoirs like Brazil are, consequently, an open door for non-native freshwater species.

Recently, the Brazilian Environmental Ministry produced a document listing 163 non-native freshwater species and their possible invasion routes (Latini et al. 2016). Among them was *Cordylophora caspia* (Pallas, 1771) (Rocha et al. 2013; Belz et al. 2016; Pereira et al. 2018), which has been causing ecological and economic imbalance in ecosystems around the world. This invasive cnidarian forms dense colonies on submerged trees, hydrothermal power plants, and piers, trapping and accumulating particulate organic matter, while creating a microhabitat for several associated species (Leppäkoski et al. 2002).

Cordylophora caspia is a colonial athecate hydroid (class Hydrozoa, family Cordylophoridae von Lendenfeld, 1885, but see "Remarks" below), cosmopolitan (Daves 1957; Rocha et al. 2013), euryhaline (Folino-Rorem and Renken 2018) found in freshwater or brackish habitats (Folino-Rorem et al. 2009; Deserti et al. 2015), and absent from fully marine environments (Schuchert 2004). The colonies of *C. caspia* are polymorphic and dioecious, with feeding (hydranths) and reproductive polyps (gonophores) (Fulton 1962; Schuchert 2004; Folino-Rorem 2015). Fertilization is internal, with male gonophores releasing sperms in the water that will fertilize mature eggs inside female gonophores (Schuchert 2004; Folino-Rorem 2015). Fertilized eggs develop into free-swimming ciliated planulae larvae that can be incubated inside the female gonophore until released in the water (Bouillon 1963; Roos 1979). The medusa stage is absent, and the larvae settle in the substrate to produce sessile, primary polyps (Bouillon 1963; Roos 1979; Folino-Rorem 2015). Bouillon (1963) observed that *C. caspia* can be either larviparous (regular life cycle) or viviparous (with the development of the larvae into polyps inside the gonophore). Asexual reproduction is also common via budding (resulting in dense branching colonies), body fragments (through mechanical disruption of hydrorhiza or hydrocauli), and via the production of resistant stages, the menonts (Roos 1979; Jormalainen et al. 1994; Folino-Rorem 2015; Pucherelli et al. 2016). Menonts are small pieces of living tissue (spheres of

coenosarc) that are protected by the outer chitinous perisarc (exoskeleton), produced under unfavorable conditions but capable of establishing new colonies when conditions are restored (Roos 1979; Folino-Rorem 2015; Deserti et al. 2015). Therefore, *C. caspia* can rapidly proliferate by different mechanisms (Darling and Folino-Rorem 2009) and asexual reproduction also contributes to local fouling and survival under unfavorable conditions (Roos 1979; Darling and Folino-Rorem 2009; Deserti et al. 2015).

The species is hypothesized to be native in the Ponto-Caspian region (the Black, Azov and Caspian Seas) (Deserti et al. 2015; Folino-Rorem and Renken 2018) and it has been introduced to several different countries, being currently recorded in all continents except Antarctica (Daves 1957; Schuchert 2004; Deserti et al. 2015; Folino-Rorem and Renken 2018). It is currently considered most likely that the invasion took place through ship ballast water or ship fouling (Folino-Rorem and Renken 2018), and it is also considered possible that spread was aided by the legs of birds (Daves 1957).

Its invasive potential is probably related to the ability to tolerate a wide range of environmental conditions, such as salinity (Folino-Rorem and Renken 2018), temperature (Folino-Rorem and Indelicato 2005), and pH (Gutierre 2012), besides anthropogenic factors such as water pollution (Hubschman 1971). The morphology of the species seems to be plastic according to the environmental conditions: low salinities usually produce spherical hydranths with short, wider and stubby tentacles, while in high salinities the hydranths are elongated and their tentacles are long and thin (Smith et al. 2002; Schuchert 2004; Folino-Rorem and Renken 2018). Freshwater specimens were observed to live up to six months without feeding (see review in Schuchert 2004).

Recent molecular analyses, including a global range of populations from freshwater and brackish habitats, resulted in multiple clades with strong correlation in salinity tolerance, possibly supporting multiple cryptic species of *Cordylophora* with different habitat preferences or a single genetically-diverse, euryhaline species (Folino-Rorem et al. 2009; Pucherelli et al. 2016). Based on this evidence, the taxonomy of the genus *Cordylophora* is in need of a detailed revision (Folino-Rorem et al. 2009; Pucherelli et al. 2016; Folino-Rorem and Renken 2018).

Because of all these characteristics related to its morphology, life cycle, and ecophysiological plasticity, *C. caspia* is becoming a predominant biofouling organism (Smith et al. 2002; Folino-Rorem and Indelicato 2005), clogging intake tunnels as well as filters and condenser tube sheets in hydroelectric power plants (Folino-Rorem and Indelicato

2005), also causing problems in the heat exchangers in the water-cooling system (Pucherelli et al. 2016), including in Brazil (Grohmann 2008). Aggregate populations of *C. caspia* can also cause damage to cement surfaces, through the formation of a dense network of filaments and possibly by metabolic processes such as the action of organic acids, or by extracting essential chemical components of cement (e.g. calcium and silicon) (Portella et al. 2009).

Despite their ecological and economic impacts, the presence of *C. caspia* in Brazil has received little attention thus far. The knowledge on the species is partially fragmented, mainly based on gray literature, such as the record for the first observation in 1991, on the Paraná river, 4km downstream from the Itaipu Dam, Paraná state (Haddad and Nakatani 1996; see also Belz et al. 2016). The species has also been recorded in São Paulo, Rio de Janeiro, and Pará states (Silveira and Boscolo 1996; Grohmann 2008; Borges et al. 2010; Belz et al. 2016). Herein, two new records of *C. caspia* in the states of Minas Gerais and Goiás are reported, characterized by a detailed morphological description and taxonomic remarks on the species, as well as the first genetic sequences (of the mitochondrial marker *Cytochrome C Oxidase Subunit I – COI*) of specimens from Brazil. Based on these sequences, we placed the Brazilian specimens in a broader phylogenetic analysis, including specimens collected at other geographic locations, discussing taxonomy and ecological signals. We also provided a brief review of the current knowledge of the species in Brazil, discussing future directions.

MATERIAL AND METHODS

Samples collection and geographic distribution

Cordylophora caspia samples were collected manually from two fish containment grids (Fig. S1 1a, b) at the hydroelectric power plant (HPP) Engenheiro José Mendes Júnior (also known as Funil), supplied by the Rio Grande river, Minas Gerais (MG) state, Brazil (-21.143139; -45.036194), and from two coal seal filters (Fig. S1 1c-e) at the HPP São Simão, Paranaíba river, Goiás (GO) state, Brazil (-19.018683; -50.500142). Samples were washed with 96% ethanol, then preserved in clean 96% ethanol and stored at -20 ºC. The authorization for activities with scientific purpose is registered in the SISBIO (Sistema de Autorização e Informação em Biodiversidade) with the access code 72222-2. The material was deposited at the Taxonomic Collections Center of the Universidade Federal de Minas Gerais, Brazil (# UFMG-INV 2000001, 2000002, 2000004, 2000005).

We reviewed the current known geographic distribution of *C. caspia* and mapped the records

using Google Earth Pro (version 7.3) to obtain the coordinates from site names described in the literature (Table 1). A map with the species distribution was constructed using SimpleMappr (Shorthouse 2010).

Morphological and cnidome analysis

The colonies were observed and photographed using a microscope Zeiss Primo Star, AxioCam ERc5s and a stereomicroscope Olympus SZX12, equipped with a camera Canon EOS SL2. Selected structures were measured using the software ImageJ (version 1.48). Observations and measurements of nematocysts were made on tissues from hydranths preserved in ethanol 96%, squashed in a drop of fresh water on a microscope slide, covered by a cover slip, and gently compressed to further dissociate the cells (Mejía-Sanchez and Marques 2013). Twenty undischarged capsules of each type of nematocyst were isolated and photographed under a microscope Zeiss Primo Star, AxioCam ERc5s and measured using Image J. Nematocysts nomenclature follows Östman (2000).

Molecular approaches

DNA extraction

Gonophores and terminal portions of tentacles from colonies of the two sampling sites were removed using sterile ophthalmic surgical instruments. Total DNA was extracted using the E.Z.N.A® Mollusc DNA Kit (Omega Bio-tek, Norcross, GA), according to the manufacturer's instructions. In order to optimize the quality of the extracted DNA, the pulverized samples with proteinase K were incubated overnight at 37 ºC in a water bath, as an alternative indicated by the kit manufacturer. DNA quantification and quality were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Polymerase chain reaction (PCR) and PCR product purification

PCR was conducted using LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ primers (Folmer et al. 1994) for the *C. caspia* mitochondrial *COI* gene which amplifies a ~710 bp (base pairs) fragment. PCR amplification was performed with ~1 ng of the extracted DNA, 12.5 µL of PCR

SuperMix (22 U/mL of recombinant Taq DNA polymerase, 22 mM Tris-HCl (pH 8,4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 µM of each dNTP, and stabilizers; Thermo Fisher Scientific, Carlsbad, CA), 1 µL of each primers at 10 µM and the volume adjusted with nuclease-free water for final reaction of 25 µL. Cycling parameters comprised an initial denaturation of 5 min (minutes) at 94 ºC, 30 rounds of amplification with 30 s (seconds) at 94 ºC, 1 min at 40 ºC, 1.5 min at 72 ºC and a final extension of 72 ºC for 5 min (Pucherelli et al. 2016). The PCR product was purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Carlsbad, CA) following the manufacturer's recommendations.

DNA sequencing and processing

DNA sequencing was performed on the 3730 DNA Analyzer (Applied Biosystems, Waltham, MA) by a commercial laboratory. The forward and reverse *COI* sequences from each sample were confirmed using the BLAST software (Basic Local Alignment Search; available at https://blast.ncbi.nlm.nih.gov/Blast.cgi) and deposited in GenBank (access numbers ON568497 and ON568498 for HPP Funil and São Simão sequences, respectively; Table S1). All sequences related to the genetic heritage of the organisms used in this study are duly in accordance with the SisGen (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado) standards and registered with the access code A118152.

Sequences obtained in this study were assembled and edited using Geneious Prime (Biomatters, Auckland, New Zealand), and then aligned with all available *Cordylophora COI* sequences (Table S1) from GenBank, as well as other anthoathecate hydroid sequences as outgroups (JN109196, *Hydractinia echinata*; EF540795, *Turritopsis rubra*; and JQ353758, *Sarsia tubulosa*), using MAFFT (Katoh et al. 2002). Missing ends of the alignments were removed based on the shortest sequence length, totaling 591 bp. Two different alignments were used for subsequent analysis including: (1) all available sequences (haplotype network and phylogenetic analysis) and (2) only unique haplotypes (ancestral state reconstruction).

Phylogenetic analysis

Phylogenetic analysis was performed based on Maximum Likelihood (ML) criteria, using PhyML 3.0 online execution tool (Guindon et al. 2010), with default settings for tree

searching. The model of nucleotide evolution GTR+G+I was selected for alignments 1 and 2 (see previous section) using the Smart Model Selection Tool (SMS, Lefort et al. 2017) available in PhyML, based on the Akaike Information Criterion (AIC). Branch support was estimated with Bootstrap based on 1,000 replicates.

Haplotype network and population genetic patterns

To evaluate patterns of genetic differentiation among *C. caspia* putative populations, a TCS network was generated based on *COI* sequences using PopART (Leigh and Bryant 2015). The number of haplotypes, haplotype and nucleotide diversity, as well as uncorrected p-distances were calculated with the packages *apex* v1.0.4 (Schliep et al. 2020) and *pegas* v1.0-1 (Paradis 2010) in R v4.1.0 (R Core Team 2020). For all these analyses, ambiguous sites were removed from the alignment (length of the alignment before/after removal: 591/585 base pairs). Finally, geographical distances in km among locations were calculated from coordinates in decimal degrees with the package *SpatialEpi* v1.2.5 (Kim et al. 2021) in R programming language.

Ancestral States Reconstruction

Habitat preferences related to salinity (freshwater or brackish) were obtained from the literature (Folino-Rorem et al. 2009; Wollschlager et al. 2013; Pucherelli et al. 2016; Table S1) and ancestral states were estimated based on Maximum Likelihood with the packages *ape* v5.5 (Paradis and Schliep 2019) and *geiger* v2.0.7 (Pennell et al. 2014) in R programming language. An ultrametric tree was generated from our ML phylogeny with the function *chronos* (*ape*), and models of morphological evolution (equal-rates-ER, symmetrical-SYM and all-rates-different-ARD) were compared (*geiger*) based on AIC values. Since the models ER and SYM had the same AIC value (15.68), followed by ARD (18.54), the ER model was selected for subsequent analyses.

RESULTS

Taxonomic Account

Class Hydrozoa Owen, 1843

Subclass Hydroidolina Collins, 2000

“Superorder Anthoathecata” Cornelius, 1992

“Order Filifera” Kühn, 1913

Family Cordylophoridae von Lendenfeld, 1885

Cordylophora caspia

Fig. 1

Cordylophora caspia.—Haddad and Nakatani 1996: 10. —Silveira and Boscolo 1996: 10. —Corbi et al. 2005: 188. —Grohmann 2008: 3, fig. 2. —Borges and Belz 2009: 111. —Portella et al. 2009: 1048, fig. 2. —Borges et al. 2010: 117. —Gutierre 2012: 92. —Belz et al. 2016: 84. —Rocha et al. 2013: Appendix A. —Pereira et al. 2018: 78. —Teixeira and Creed 2020: 34, 39, tables S2, S3.

Cordylophora sp. Roque et al. 2004: 6. — da Silva Bertão et al. 2021: 5174, Table 1.

Material examined

UFMG-INV 2000001, colony, HPP Engenheiro José Mendes Júnior, Minas Gerais state, Rio Grande river, Brazil (-21.143139; -45.036194), 10 June 2019, on fish containment grid (G1), ethanol 96%, collected by N.P.U. Barbosa and G. Bretas, identified by L.S. Miranda. UFMG-INV 2000002, colony, HPP Engenheiro José Mendes Júnior, Minas Gerais state, Rio Grande river, Brazil (-21.143139; -45.036194), 10 June 2019, on fish containment grid (G2), ethanol 96%, collected by N.P.U. Barbosa and G. Bretas, identified by L.S. Miranda. UFMG-INV 2000004, colony, HPP São Simão, Goiás state, Paranaíba river, Brazil (-19.018683; -50.500142), 19 March 2020, on coal seal filter (right, F1), ethanol 96%, collected by A.L. Pinto, identified by L.S. Miranda. UFMG-INV 2000005, colony, HPP São Simão, Goiás state, Paranaíba river, Brazil (-19.018683; -50.500142), 19 March 2020, on coal seal filter (left, F2), ethanol 96%, collected by A.L. Pinto, identified by L.S. Miranda.

Description

Colonies erect, monosiphonic, up to 2.5 cm high. First order branches only, irregularly disposed at different angles (Fig. 1a), but several unbranched stems arising from a dense

network of stolons (Fig. 1b–c), some presumably autoepizoic. Annulations at base of stems (up to 10) and branches (4 to 5), but some branches irregularly annulated throughout (Fig. 1d). Shape of hydranths variable, usually ovoid (as a result of contraction), but some are taller and narrower (compare Fig. 1d–e and 1f–g, Table 2). Tentacles filiform, 12 to 18 in number, scattered over hydranth body but roughly concentrated on medium to distal part (Fig. 1d–g). Hydranths with two types of nematocysts: desmoneme (more abundant) and microbasic eurytele (Table 3). Colonies dioecious, gonophores arising from pedicels of hydranths, branches and stem. Gonophores ovoid, male gonophores 459.66 µm long and 373.36 µm wide in average (Fig. 1h), female gonophores 779.83 µm long and 563.64 µm wide in average, with branched spadix (Fig. 1i–j). Female gonophores with up to 11 oocytes, each 143.29 µm wide in average, and internal fertilization inferred from the presence of planulae (Fig. 1k).

Remarks

Phylogenetic relationships are still controversial among the non-monophyletic Anthoathecata, and this is reflected in the taxonomic history of the genus *Cordylophora* Allman, 1843. Diagnosis at the family level within Filifera is often ambiguous due to a paucity of morphological characters, with several families characterized by the presence of filiform tentacles scattered over hydranths (Schuchert 2001, 2004). As a result, *Cordylophora* has been historically assigned to either Clavidae McCrady, 1859, Oceanidae Eschscholtz, 1829, Cordylophoridae von Lendenfeld, 1885 or Bougainvilliidae Lütken, 1850 (Calder 1988, 2010, 2019; Schuchert 2001, 2004, 2012), and its phylogenetic position is still in discussion. Results from molecular studies recovered *Cordylophora* as part of Bougainvilliidae, but this family was also not supported as monophyletic (Cartwright et al. 2008; Mendoza-Becerril et al. 2018). Here we follow Calder (2010, 2019) and Schuchert (2001, 2022) and assign *Cordylophora* to the family Cordylophoridae von Lendenfeld, 1885, although this decision is provisional and should be confirmed with additional molecular and morphological studies. While Cordylophoridae is characterized by the presence of scattered filiform tentacles and the absence of a pseudohydrotheca, with perisarc ending below hydranths (Schuchert 2001; Calder 2010), new evidences indicate that *Cordylophora* is part of the clade Pseudothecata (Mendoza-Becerril et al. 2018), which is supported by the presence of exosarc over hydranths (Mendoza-Becerril et al. 2016, 2018). This suggests that histological characters of the exoskeleton are promising and could

contribute to the delimitation of families within Pseudothecata (e.g., Mendoza-Becerril et al. 2017), including *Cordylophoridae*.

At the genus level, *Cordylophora* is usually distinguished by having tentacles scattered mainly over the distal half of hydranth body, as opposed to scattered over most of hydranth as in *Turritopsis* McCrady, 1857 or in two or more whorls as in *Pachycordyle* Weismann, 1883 (Calder 2010; Schuchert 2012). The presence of a branched spadix in the gonophores is also diagnostic of the genus (Calder 2010; Schuchert 2012). These characters, however, are not easily discernible, and of the 14 nominal *Cordylophora* species originally described (Folino-Rorem 2009), four have later been transferred to or synonymized with species of *Pachycordyle* (i.e., *P. napolitana*, *P. pusilla*, *P. mashikoi* and *P. navis=P. michaeli*; Calder 1988; Schuchert 2004, 2007; Stepanjants et al. 2000). Similarly, all remaining species are currently considered synonyms of *C. caspia*, with the exception of *C. solangiae* (Folino-Rorem 2009; Schuchert 2004, 2022), originally recorded from the Tuamotu Archipelago, French Polynesia (Redier 1967).

Species delimitation in *Cordylophora* is often complicated because of morphological plasticity (see Folino 1999; Folino-Rorem 2009). Several nominal species were originally described based on variations of characters such as colony size, branching, number of annulations and tentacles, shape and number of gonophores (e.g., *C. lacustris* Allman, 1844, *C. albicola* Kirchenpauer in Busk, 1861, *C. americana* Leidy, 1870, *C. whiteleggi* von Lendenfeld, 1887, *C. otagoensis* Fyfe, 1929, *C. japonica* Itô, 1951) (Busk 1861; Leidy 1870; Allman 1871: 252; Lendenfeld 1887; Itô 1951, 1952; Fyfe 1929). However, most of these characters were later shown to vary with salinity in *C. caspia* (Kinne 1971; Schuchert 2004; Folino-Rorem and Renken 2018). Similarly, nematocyst size has not proven useful to separate the species (Wollschlager et al. 2013; Table 3). Presently, the two valid species are mainly differentiated by the number of tentacles and habitat preferences, with *C. solangiae* being found in fully marine environments (Redier 1967; Table 2).

World distribution and habitat

The species is cosmopolitan and absent from fully marine environments, with records in all continents except Antarctica, in both freshwater and brackish habitats (see Folino-Rorem 2009).

Distribution and habitat in Brazil

Besides the two new records at the hydroelectric power plant (HPP) Engenheiro José Mendes Júnior (also known as Funil), Minas Gerais state, Rio Grande river, Brazil (-21.143139; -45.036194), and at HPP São Simão, Goiás state, Paranaíba river, Brazil (-19.018683; -50.500142), *C. caspia* has been already recorded in different hydroelectric power plants in Paraná, Rio de Janeiro, São Paulo, and Pará states (Table 1; Fig. 2). There is also a record in the estuary of Rio Escuro, Ubatuba, São Paulo state (natural environment) and another record at Paraná river, Três Lagoas, Mato Grosso do Sul state (no information whether the sample was collected in natural or artificial environment) (Table 1; Fig. 2). Based on these records, we assume that *C. caspia* in Brazil can be found in both brackish and freshwater environments. *Cordylophora caspia* has already been recorded as a host of Oligochaeta (Corbi et al. 2005) and Chironomidae larvae (Roque et al. 2004).

Evolutionary analysis

We retrieved three main clades in our ML tree based on *COI*, herein clade 1, clade 2 and clade 3 (Fig. 3, following Folino-Rorem et al. 2009). Clade 1 is composed mainly of freshwater samples and can be subdivided into two main clades. Clade 1A is composed exclusively of freshwater samples from Panama and Eastern and Western USA. Clade 1B is composed mostly of freshwater samples from Hungary and Western USA, but also brackish colonies from Eastern USA (Fig. 3). Clade 2 is composed mainly of brackish samples and can be subdivided into two main clades. Clade 2A is composed exclusively of brackish samples from different regions around the world: Chile, France, Germany, Ireland, and Eastern USA. Clade 2B is composed exclusively of brackish samples from Western USA. Finally, clade 3 is composed exclusively of our freshwater samples from Brazil. The phylogenetic placement of Brazilian material into clade 3 was supported by high bootstrap value (100%), but its phylogenetic position as sister group to clade 2 is weakly supported (~70%) and could result in a different configuration with the inclusion of more samples and molecular markers in future analyses.

Measures of haplotype and nucleotide diversity of populations of *C. caspia* were generally high (0.78 and 0.08, respectively), with only a few shared haplotypes among western USA, eastern USA, and Panama (H1, Fig. S2, Table S1), as well as among eastern USA, France, Germany, and Ireland (H2, Fig. S2, Table S1), as in Folino-Rorem et al. (2009). Samples from Brazil represent a unique haplotype, shared by specimens from HPP Funil and HPP

São Simão, which indicates low genetic diversity among Brazilian populations that occur at least 530 km apart (euclidean distance between sampled locations). In addition, samples from Brazil showed considerable genetic distance of *COI* sequences when compared to other clades and subclades of the phylogeny (Table 4), with a minimum distance between clade 3 and any other clade of 14.36%. The second highest minimum genetic distance was observed between clades 1 and 2 (12.65%), followed by clades 2A and 2B, and clades 1A and 1B (9.74% and 7.86%, respectively). Considering the different subclades of the phylogeny, clade 2A showed the highest maximum intra-clade distance (6.32%, Table 4) related to the sequence from Chile, with a mean intra-clade genetic p-distance of 2.53%. The second highest maximum intra-clade distance shown by clade 1B was considerably smaller (1.71%).

Ancestral state reconstruction analysis supports a moderately higher probability (63%) that the ancestral of *C. caspia*, among the current sampled populations, was present in the freshwater environment (Fig. 4). Additionally, two independent shifts occurred between fresh and brackish waters: in clade 1B and in clade 2+3 (Fig. 4). While in clade 1B, the brackish lineage represented by haplotype H8 probably derived secondarily from a freshwater ancestor, in clade 3 the opposite scenario was observed (Fig. 4). The ancestor of clade 2+3 had a moderately higher probability (63%) of its presence in brackish waters, and the Brazilian freshwater lineage (clade 3) most likely represents a recolonization of this environment by *C. caspia* (Fig. 4).

DISCUSSION

Cordylophora caspia is an invasive species that causes environmental and economic impacts (Leppäkoski et al. 2002; Obolewski el al. 2015). For this reason, it is crucial to monitor and update its geographic distribution around the world in order to define mitigation strategies to prevent and control the invasion process (Vander Zanden and Olden 2008). Here we reviewed and updated the current distribution of *C. caspia* in Brazil, reporting two new sites of occurrences, the first for Minas Gerais and Goiás states. Furthermore, we described specimens of *C. caspia* based on morphological and molecular data, providing the first genetic sequences for this invader in Brazilian waters. Although there are few records in Brazil and many of them were provided in gray literature (Table 1; Fig. 2), the known geographic distribution of *C. caspia* in the country encompasses different regions, suggesting a potential broad distribution. Most of the

reports are in the South and Southeast regions, probably because of collection bias in these areas, which are more frequently studied (see Pereira et al. 2018). There is a huge gap between these records (including also those in the Central West region) and the record in the North region, in Pará state (Table 1; Fig. 2), which could indicate that the species has been overlooked and/or undersampled in different states. Indeed, *C. caspia* gathers different biological and ecological aspects that make them excellent invaders: it can alternate between asexual and sexual reproductions (Darling and Folino-Rorem 2009) and also produce menonts, pieces of living tissues that can persevere during unfavorable conditions and establish new colonies when conditions are suitable (Roos 1979; Folino-Rorem 2015; Deserti et al. 2015); it has a broad ecophysiological tolerance (Rajagopal et al. 2002; Wintzer et al. 2011; Meek et al. 2012; Folino-Rorem and Renken 2018); and it can occupy the niche of a benthic colonial predator, affecting the local food webs after invasion (Smith et al. 2002; Berg and Folino-Rorem 2009). All these characteristics reinforce the potential for a broad distribution of *C. caspia* in Brazil, and most concerning, a highly underestimated expansion of its area of invasion, considering the most recent accounts on the occurrence of the species in Brazilian continental waters (Latini et al. 2016; Pereira et al. 2018).

Genetic diversity of C. caspia and inferences on salinity preferences

In a previous study, Folino-Rorem et al. (2009) suggested that *Cordylophora* might comprise multiple cryptic species, which could support traditional arguments that *C. caspia* (Pallas, 1771) and *C. lacustris* Allman, 1844 are valid species with brackish and freshwater preferences, respectively (see Folino 1999; Schuchert 2004). However, the phylogenetic placement of our Brazilian clade 3 do not support the independent species *C. caspia* and *C. lacustris* based on salinity preferences, since it did not cluster with the freshwater lineage (clade 1) as would be expected in that scenario (Fig. 4). Similarly, the high genetic differentiation within *Cordylophora* lineages observed in that study suggested a role for multiple introductions from genetically divergent sources to the same non-native sites (Folino-Rorem et al. 2009). Our samples corroborate this pattern, showing high genetic distances (minimum inter-clade distances over 14%, Table 4) of Brazilian clade 3 compared to previously proposed clades 1 and 2 (Folino-Rorem et al. 2009). These levels of differentiation are comparable to recent studies using *COI* to unravel genetic diversity among *Pteroclava* and *Zanclea* species (minimum inter-clade

distances of 9.3% and 19.7%, respectively; Montano et al. 2017; Maggioni et al. 2020), and could indicate at least a third putative species in the *C. caspia* species complex. Nevertheless, currently there is no morphological or ecophysiological evidence that would justify and support the subdivision of *C. caspia* in different species. Additional genetic information from different localities in Brazil will be important to determine the possibility of multiple introductions of independent lineages of *Cordylophora* in Brazilian continental waters.

At the population level, high genetic differentiation could also be a result of limited dispersal ability of *Cordylophora*, leading to *in situ* population differentiation after initial introduction (Darling and Folino-Rorem 2009). In fact, evidence of isolation by distance among hydroid populations is recurrent (e.g., Postaire et al. 2017a, 2017b), and was also observed among *Cordylophora* populations in the Great Lakes Basin (Darling and Folino-Rorem 2009). High genetic differentiation over small spatial scales is particularly common among benthic species such as *C. caspia*, which lacks a long-lived planktonic stage (Cunha et al. 2016; Postaire et al. 2016; Boissin et al. 2018;). Studies show that planula larvae of *C. caspia* lives in the plankton for only 12 to 24 hours, while larvae liberated from benthic hydroids in general usually settles near the mother colony, where suitable substrate is available (Sommer 1992; Gili and Hughes 1995). These life history traits presumably constrain dispersal, and could account in the long-term for the high genetic differentiation observed among *Cordylophora* lineages. On the other hand, dispersal of asexually produced propagules has been reported as an important trait linked to higher invasion success of freshwater species, and could facilitate population establishment and subsequent spread of species with ability to reproduce sexually and asexually, such as *C. caspia* (Pigneur et al. 2014; Eckert et al. 2016; Casties and Briski 2019). Although asexual propagules were not found to contribute to the spatial spread of *Cordylophora* populations in the Great Lakes basin (Darling and Folino-Rorem 2009), Brazilian *C. caspia* from Minas Gerais and Goiás states consisted of the same *COI* haplotype sampled in reservoirs located at least 530km apart, suggesting a possible role of asexual propagules and/or resting stages (menonts) in dispersal. Nonetheless, data from additional molecular markers will be necessary to determine if samples from these two populations indeed correspond to true clonal genotypes, as evidence of sexual reproduction (i.e., gonophores and larvae) were observed in both Brazilian populations analyzed.

Inland range expansion of *C. caspia* has been frequently attributed to its ability to tolerate

wide variations in salinity (Folino-Rorem et al. 2009; Pucherelli et al. 2016), although these studies have not been able to fully determine whether the ability of the species to acclimate precedes or follows range expansion through human-mediated introductions (Folino-Rorem et al. 2009; also see Lee and Bell 1999; Strayer 1999; Wolff 2000). Among the possible scenarios, the *Cordylophora* lineage was hypothesized to have brackish water ancestry, with freshwater populations arising more recently through adaptation after expansion to freshwater habitats (Folino-Rorem et al. 2009). However, our ancestral reconstruction analysis of the trait salinity, based on the available sequence sampling (not all sequences obtained by and used in Folino-Rorem et al. (2009) were available in GenBank), suggests a different scenario, in which the ancestral *Cordylophora* was more likely adapted to live in freshwater, with derived and independent shifts to brackish waters. This is consistent with results from recent studies that suggest a freshwater euryhaline ancestry for invasive species native to the Ponto-Caspian region (the Black, Azov and Caspian Seas), such as *C. caspia* (Casties et al. 2016; Paiva et al. 2018; Pauli and Briski 2018). In fact, Pauli and Briski (2018) showed that *C. caspia* have shifted its salinity range from native to introduced habitats, expanding its range in both directions (towards lower and higher salinities). In addition, studies have shown that this ability to tolerate broad variations in salinity is typical of Ponto-Caspian invasive species and probably a result of the fluctuations in salinity historically experienced in their native habitat (Pauli and Briski 2018; Paiva et al. 2018; Cuthbert et al. 2020). Similarly, while different populations may have different adaptation potential to varying salinity (as shown for brackish and freshwater populations of *C. caspia*, Folino-Rorem and Renken 2018), Ponto-Caspian taxa tend to have higher tolerance and adaptability to lower salinity, which may explain their high invasive potential in freshwater habitats and further support their freshwater origins (Paiva et al. 2018; Pauli et al. 2018). A presumed freshwater origin for *C. caspia*, in addition to its genetic differentiation patterns shown in this study, suggests a high probability of the species for rapid adaptation to new freshwater environments and reinforces its invasive potential in Brazilian continental waters, especially considering it has mostly been found in hydroelectric power plants in Brazil. Additional studies are therefore critical to better assess the species invasive potential and adaptability to fluctuating environments, especially regarding temporal and spatial variations in salinity.

Ecological interactions of invasive species, economic impacts, and antifouling strategies

The ecological relationship established between *C. caspia* and invading mollusks, such as zebra and quagga mussels, has been shown to be quite complex and dual. Quagga mussel larvae have been found in feeding polyps of *C. caspia* (Pucherelli et al. 2016), whereas these mussels used the hydroid filaments as substrate for their settlement (Moreteau and Khalanski, 1994). *Cordylophora caspia* colonizes different types of substrates, including rocks, plant stalks, and also bivalve shells, including zebra mussel (Curry et al. 1981; Olenin and Leppäkoski 1999).

In Brazil, the association between *C. caspia* and the invading golden mussel *Limnoperna fortunei* was reported at the Governador José Richa hydroelectric power plant (HPP Gov. José Richa; Table 1; Fig. 2), in the state of Paraná, where the colonization of cement slabs by these two invading organisms was evaluated during one year (Portella et al. 2009). Initially, the plates were colonized only by *C. caspia*, then followed by a late colonization by golden mussel in exposed areas of the plaques caused by previous leaching by *C. caspia* (Portella et al. 2009). *Limnoperna fortunei* adherence was also observed over *C. caspia* colonies previously settled (Portella et al. 2009). This interaction between the golden mussel and *C. caspia* reinforces the importance of mapping and early detecting the presence of these invaders in the environment (Vander Zanden and Olden 2008), since the presence of both organisms can favor the biofouling of each other. Specifically, we observed the association between *L. fortunei* and *C. caspia* in samples collected from HPP São Simão (Fig. S3; Fig. 2; Table 1) highlighting the importance of a correct identification of these species in order to define the management, control, and combat strategies contemplating both invasive species (Resende and Martinez 2008). On the other hand, at HPP Engº José Mendes Júnior (Funil) (Fig. 2; Table 1), we only detected *C. caspia*, which could be considered as an alert sign, since the occurrence of *C. caspia* seems to precede the occurrence of *L. fortunei* (Portella et al. 2009).

Recently, ecological interactions between invasive and native organisms were evaluated in order to define the best period of year to employ antifouling strategies from pipes of HPP Gov. José Richa (da Silva Bertão et al. 2021). The results revealed that late spring and early summer were the adequate periods because the density of fouling organisms was higher in the polystyrene plates seated to the log boom located near to the dam (da Silva Bertão et al. 2021). The complex interaction between *C. caspia* (as *Cordylophora* sp.) and *L. fortunei* was highlighted: the two species compete for food and habitat,

especially when the settlement area was limited, although *C. caspia* also served as substrate for *L. fortunei* (epibiosis). Apparently, golden mussel larvae fix on *C. caspia* colonies to avoid local hydrodynamics, a behavior previously reported between *C. caspia* and *Dreissena polymorpha* (Pallas, 1771) (Folino-Rorem et al. 2006). *Cordylophora caspia* and *L. fortunei* were also reported to interact mutualistically during settlement, which can amplify the invasion impact, although *C. caspia* can also predate *L. fortunei* (da Silva Bertão et al. 2021). Understanding the role of each organism in different and dynamic ecological interactions can provide valuable tools to understand the environmental and economic damage caused by the presence of these species (Green et al. 2011; Wegner et al. 2019).

Invasive species are widely studied because of the economic and ecological problems they cause in many environments (Deserti et al. 2015). Despite their impacts, strategies for controlling these species are generally nonspecific. *Cordylophora caspia* is specially complicated to deal with due to its wide range of resistance to environmental changes. For example, specimens can tolerate a range from 8° C to more than 30° C (Fulton 1962; Folino-Rorem and Indelicato 2005), have a high survival rate within pH range of 5.0– 8.5 (Gutierre 2012), and can proliferate in salinity range from 0–40 PSU (Folino-Rorem et al. 2009). On the other hand, high levels of O_2 dissolved in water seems to be essential to its maintenance in the environment (Fulton 1962). Hydro-optic ultraviolet light (Pucherelli et al. 2018) and chlorine (Rajagopal et al. 2002; Mant et al. 2012) were tested to control biofouling by *C. caspia* with different degrees of success, although resistance of surviving colonies has been highlighted (Mant et al. 2012). Furthermore, the possible presence of *C. caspia* regenerative tissues (menonts) suspended in the water column is an additional factor that hinders the viability of the treatments and the control of their dispersion (Folino-Rorem and Indelicato 2005). *Cordylophora caspia* great adaptive capacity forces the use of aggressive treatments to control the infestation, although these strategies still have low applicability in open environments such as reservoirs (Rajagopal et al. 2002; Folino-Rorem and Indelicato 2005; Mant et al. 2012). Promoting integrative research focused on the species, including data on morphology, life cycle, evolution, and ecophysiology, besides raising public awareness of its occurrence in Brazilian waters, is possibly the best strategy to detect and control *C. caspia* invasion.

CONCLUSIONS

Recognizing an invasive species in the environment is extremely important for the adoption of containment measures to prevent its spread, as well as applying efficient control and combat methods. Although the presence of *C. caspia* is documented in the Brazilian territory, with associated reports of economic damage in hydroelectric power plants, its current geographic distribution in the country is probably underestimated. The species is usually mistaken for macrophytes or algae by hydroelectric dam managers in Brazil due to its filamentous aspect. This lack of knowledge of the species distribution limits the understanding of its biological and ecological aspects. In this study, we reviewed the current records of *C. caspia* for Brazilian continental waters, and reported two new records for Minas Gerais and Goiás states, expanding its known geographic distribution in Brazil. Besides, we included Brazilian samples in an evolutionary perspective for the first time. Considering the species genetic patterns, we found no evidence for validation of the junior subjective synonym *C. lacustris* based on salinity preferences, although the high genetic levels of differentiation of Brazilian samples suggest they might constitute an independent, cryptic species. Genetic data provided in this study, associated with further molecular, morphological and life cycle evidence, are crucial for establishing hypotheses regarding species delimitation within *Cordylophora* and for discussing invasive routes around the world.

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Authors contribution

RSdP, AFC, AVC, ECJ, and LSM contributed to the study conceptualization, provided resources, and acquired funding for the study. RSdP, AFC, MPR, and LSM developed the study methodology; NPUB collected data; RSdP, AFC, MPR, CCS, RBOJ, NPUB, and LSM ran analyses and visualized the results. RSdP, MPR, and CCS wrote the first draft of the manuscript and AFC, AVC, ECJ, and LSM reviewed, edited, and made intellectual

contributions to subsequent revisions of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability

The *COI* sequences of *Cordylophora* generated in this work were deposited in GenBank and will be available after the publication of this article with the respective Accession Numbers specified in the Methodology section. All other data generated or analyzed during this study are included in this published article (and its online resource information files).

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FIGURES LEGENDS

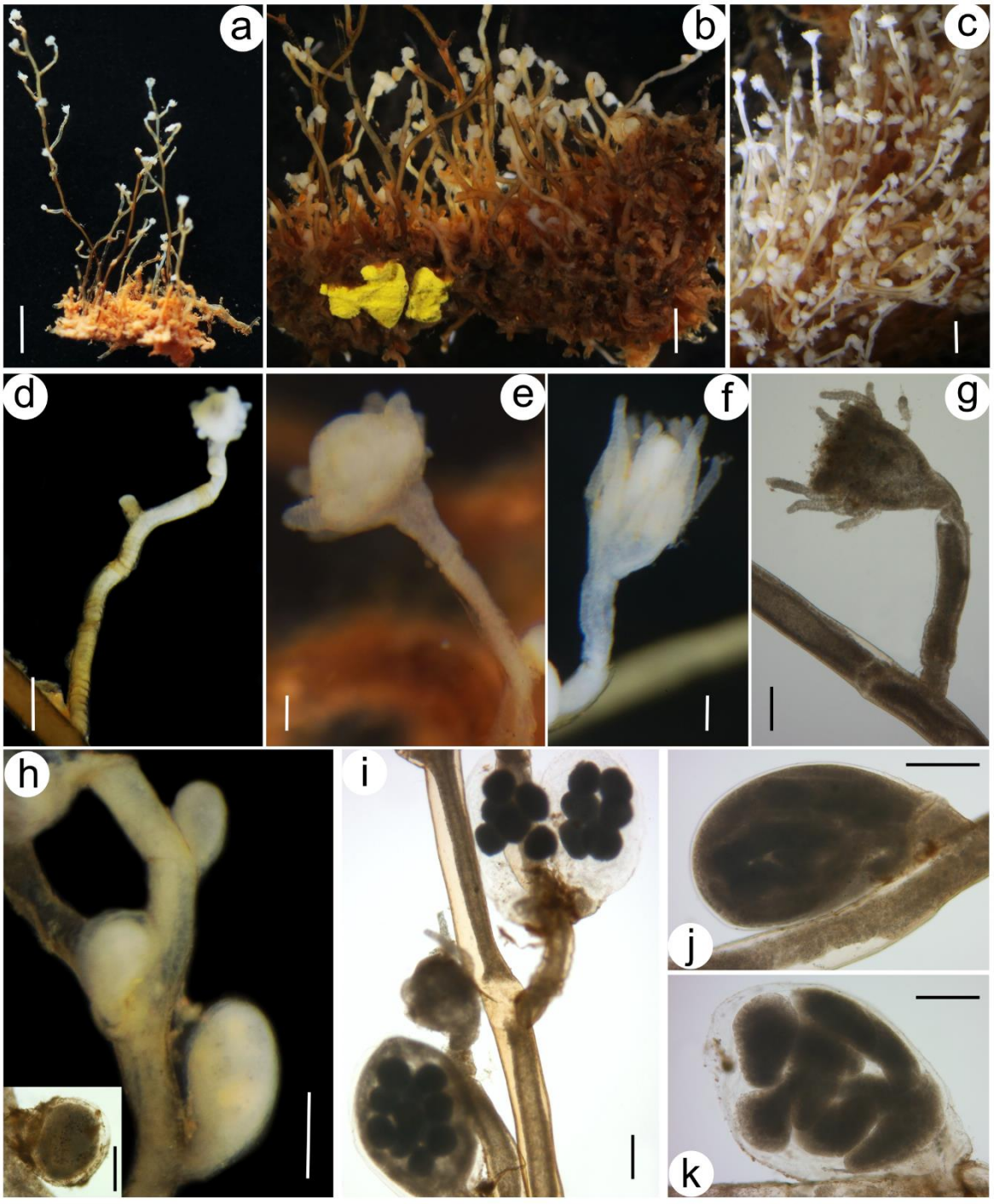

Fig 1. Morphology of *Cordylophora caspia* sampled at hydroelectric power plants. (a-b) and (i-k) samples from Engenheiro José Mendes Júnior Hydroelectric Power Plant (HPP

Funil); (c-h), samples from HPP São Simão. Scales: (a) 2 mm; (b, c) 800 µm; (d) 500 µm; (e-f) 100 µm; (g, i-k) 200 µm; (h) 400 µm. For more details, see Results.

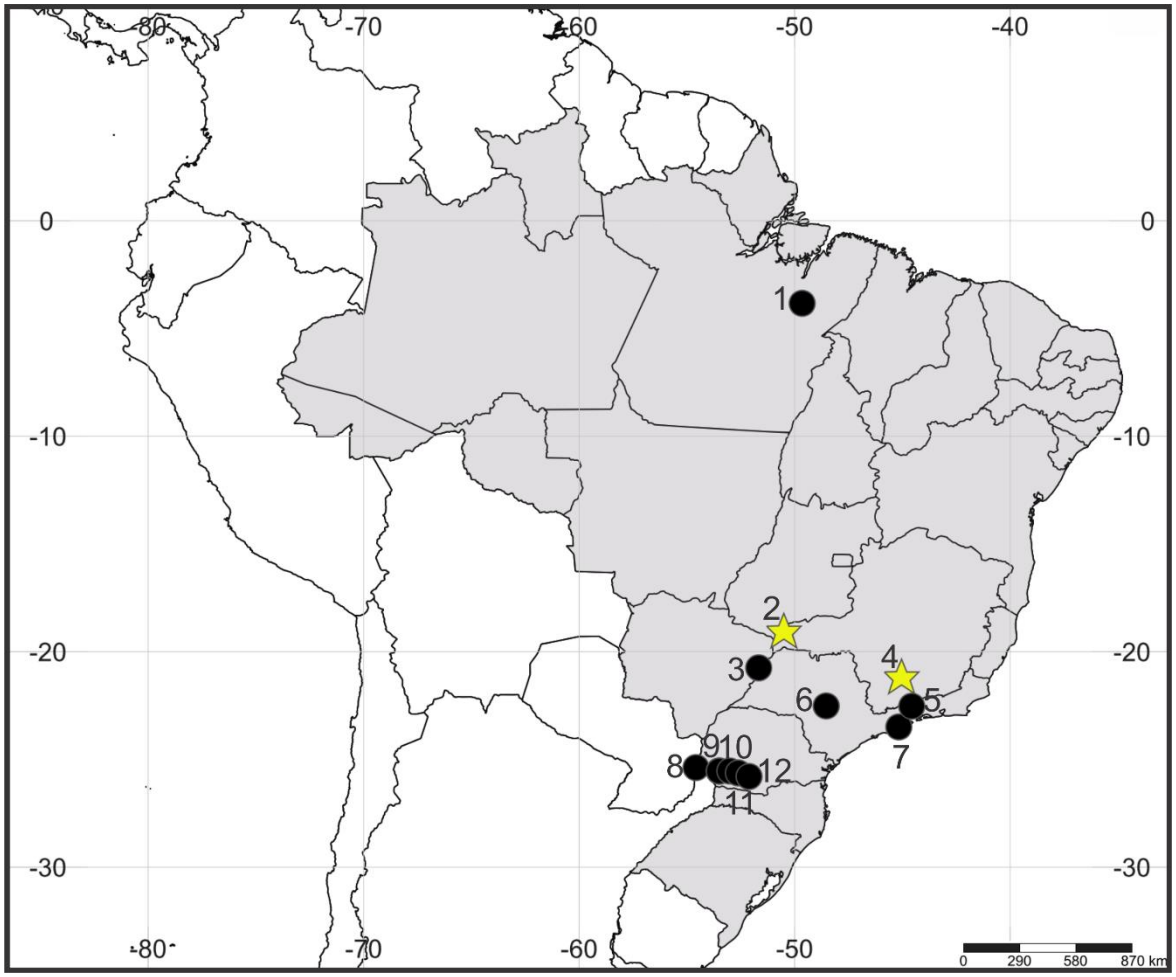

Fig 2. Map of distribution of *Cordylophora caspia* in Brazil. (a) Review of the distribution of *C. caspia* in Brazil (Table 1), with new records at Engenheiro José Mendes Júnior Hydroelectric Power Plant (HPP Funil), Minas Gerais, and at São Simão Hydroelectric Power Plant (HPP São Simão), Goiás, highlighted with a yellow star. 1) HPP Tucuruí, PA; 2) HPP São Simão, GO; 3) Três Lagoas, MS; 4) HPP Eng.º José Mendes Júnior (Funil), MG; 5) HPP Funil, RJ; 6) HPP Barra Bonita, SP; 7) Rio Escuro, Ubatuba, SP; 8) HPP de Itaipu, PR; 9) HPP Gov. José Richa (Salto Caxias), PR; 10) HPP Salto Osório, PR; 11) HPP Salto Santiago, PR; 12) HPP Gov. Ney Braga de Barros (Segredo), PR.

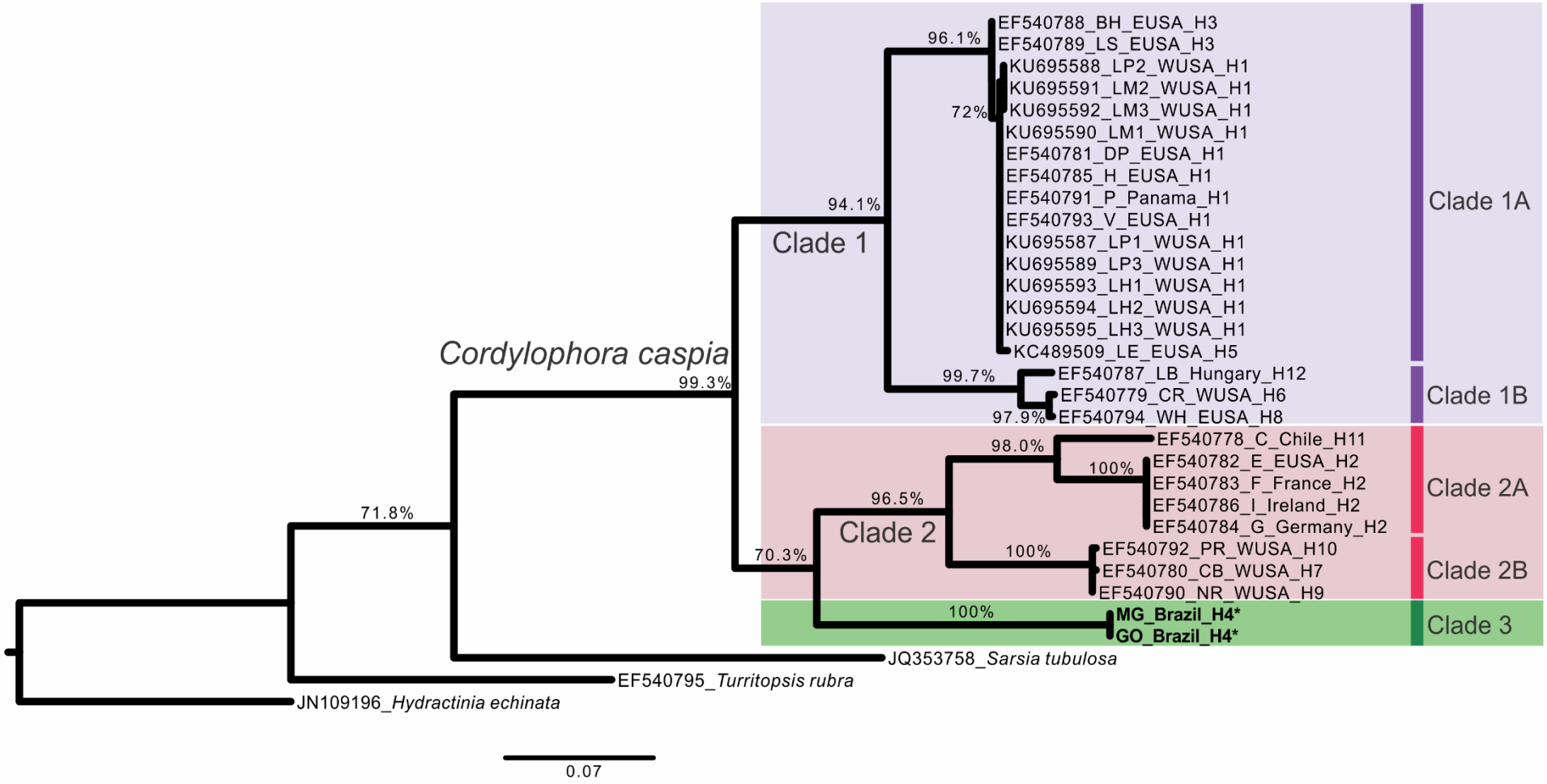

Fig 3. Maximum likelihood (ML) phylogenetic hypothesis based on *COI*. Bootstrap values are shown for each node, indicated as percent of 1,000 replicates. Clade names are based on Folino-Rorem et al. (2009). The sequences comprise 12 different haplotypes (codes H1 to H12), and terminals with the same code indicate individuals sharing the same haplotype (see Table S1 for additional information). *Sequences from this study.

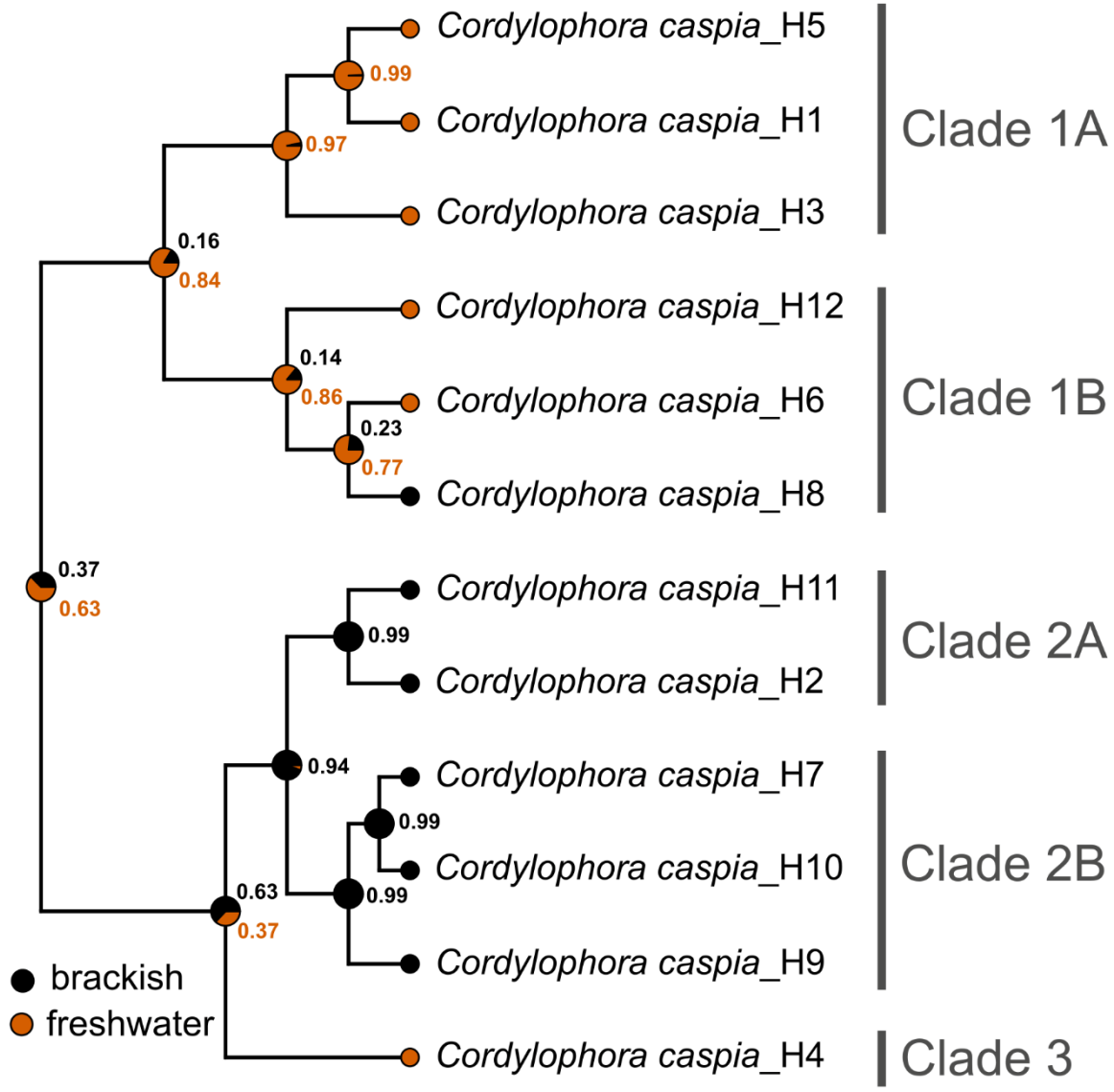

Fig 4. Ancestral state reconstruction of habitat preferences of *Cordylophora caspia* based on salinity. Pie diagrams and numbers at nodes indicate proportional likelihoods of each state, with freshwater (orange) or brackish (black) habitats. See Table S1 for additional information and references.

SUPPLEMENTARY FIGURES

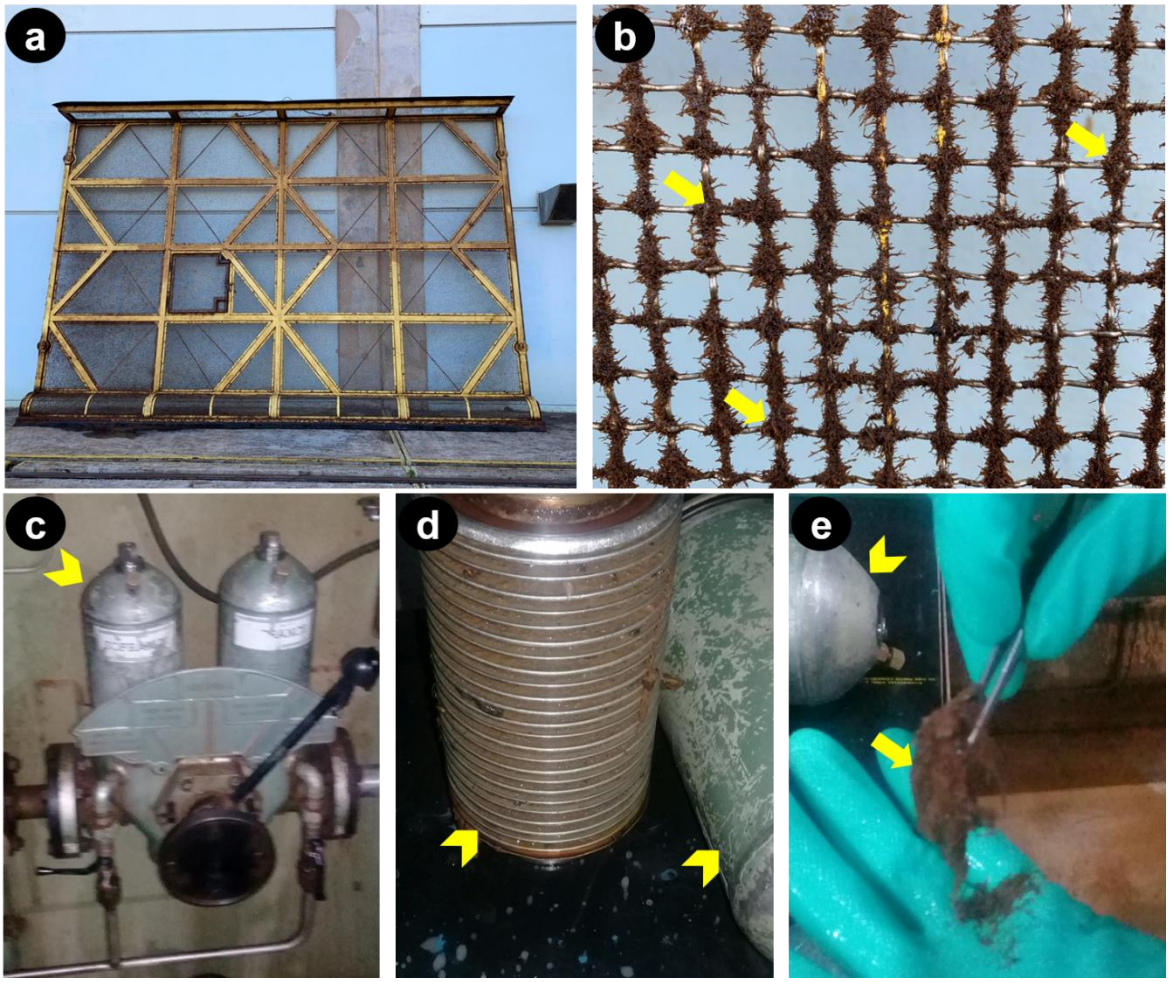

Fig S1. Samples used in this study. (a) Fish containment grid from Engenheiro José Mendes Júnior Hydroelectric Power Plant (HPP Funil). (b) Fish containment grid in detail with biofouling caused by *C. caspia* (yellow arrows). (c) Coal seal filter (yellow arrowhead) of the hydraulic circulation system of the HPP São Simão where *C. caspia* has been sampled. (d) Coal seal filter in detail. (e) *C. caspia* (yellow arrow) sampled from coal seal filters.

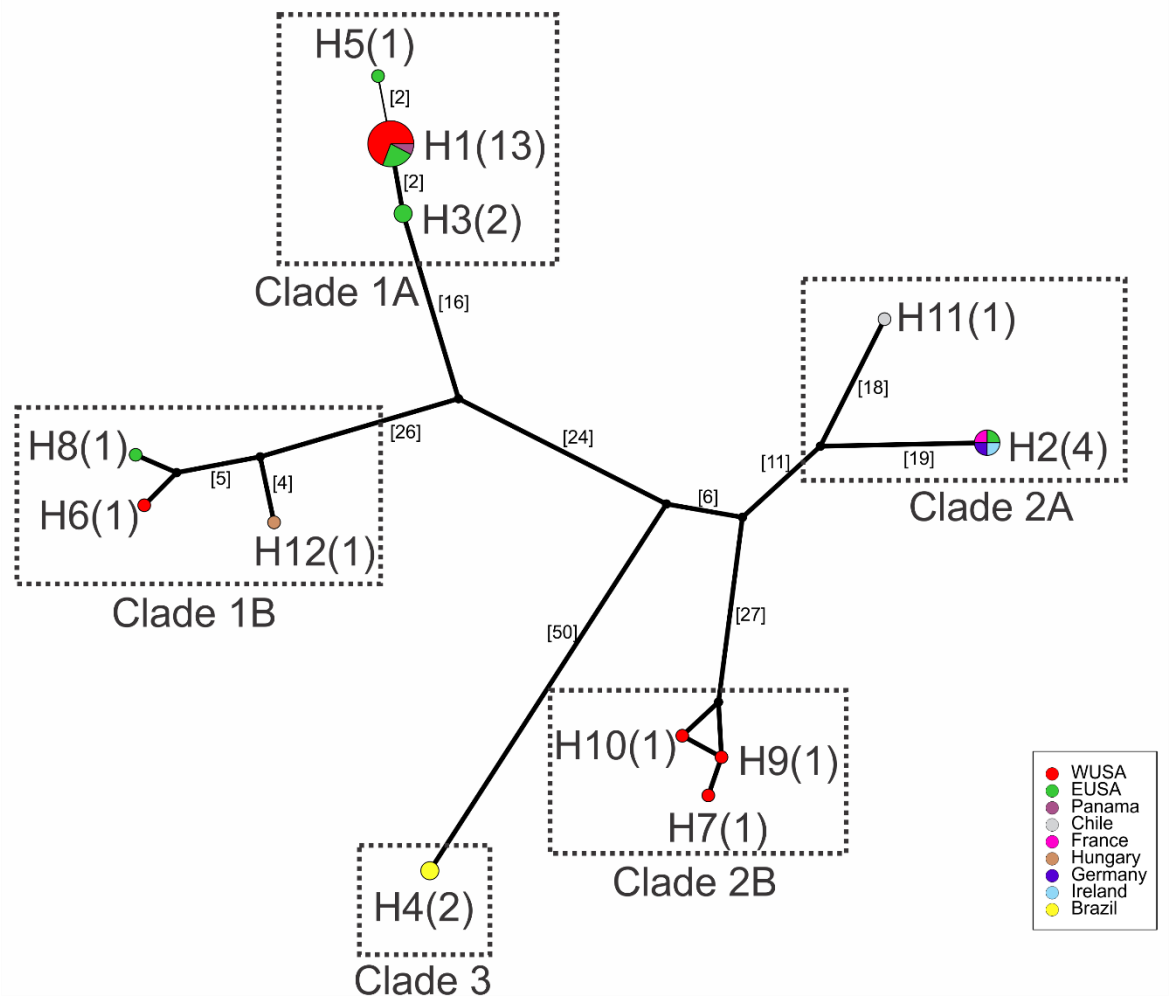

Fig S2. TCS haplotype network for *Cordylophora caspia* based on *COI* sequences. Circle sizes are proportional to the number of individuals with each haplotype (indicated in parenthesis), and distances between circles correspond to the number of mutations between haplotypes (if not indicated, distance equals 1). WUSA: West of the United States of America; EUSA: East of the United States of America.

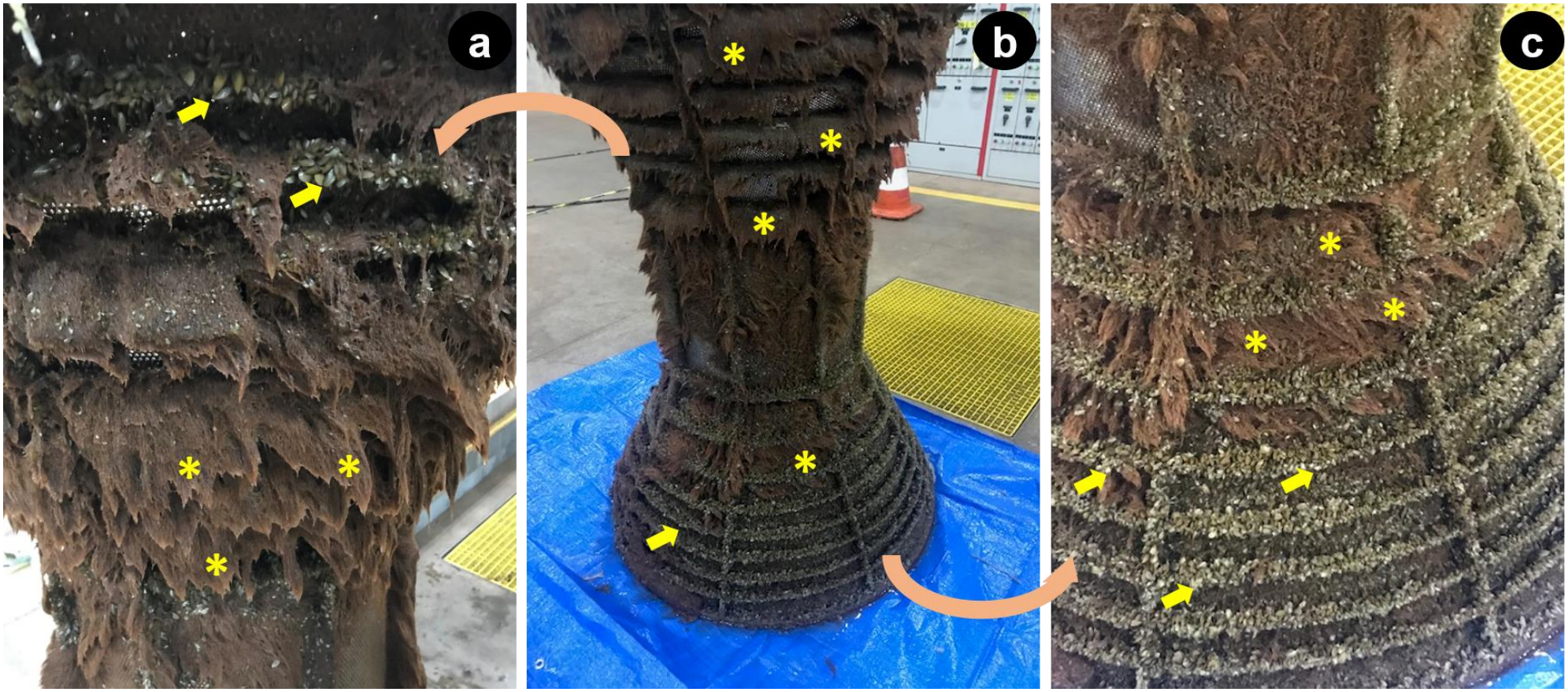

Fig S3. Concomitant macrofouling of *Cordylophora caspia* and *Limnoperna fortunei* in the coarse filter of the HPP São Simão refrigeration system. (a), (b) and (c) Coarse filter of the cooling system. (a) filter top shown in (b); (c) lower part of the filter represented in (b). Asterisks: *C. caspia* macrofouling; yellow arrows: *L. fortunei*.

Tables

Table 1. Review of the geographic distribution of *Cordylophora caspia* in Brazil. States: PA, Pará; GO, Goiás; MS, Mato Grosso do Sul; MG, Minas Gerais; RJ, Rio de Janeiro; SP, São Paulo; PR, Paraná. HPP= Hydroelectric Power Plant.

Locality	Latitude	Longitude	Environment	References
HPP de Tucuruí, Tocantins river, PA	-3.833254	-49.649774	hydroelectric power plant	Belz et al. 2016
HPP São Simão, Paranaíba river, GO	-19.018683	-50.500142	hydroelectric power plant	**this study**
Três Lagoas, Paraná river, MS	-20.750000	-51.666667	not informed	Roque et al. 2004
HPP Eng.º José Mendes Júnior (Funil), Rio Grande river, MG	-21.143139	-45.036194	hydroelectric power plant	**this study**
HPP de Funil, Paraíba do Sul river, RJ	-22.530213	-44.564161	hydroelectric power plant	Grohmann 2008
HPP Barra Bonita, Tietê river, SP	-22.51973	-48.533905	hydroelectric power plant	Borges et al. 2010
Rio Escuro, Ubatuba, SP	-23.493534	-45.165528	estuary	Silveira and Boscolo 1996
HPP de Itaipu, Paraná river, PR	-25.380154	-54.575871	hydroelectric power plant	Haddad and Nakatani 1996; Belz et al. 2016
HPP Gov. José Richa (Salto Caxias), Iguaçu river, PR	-25.541667	-53.497500	hydroelectric power plant	Portella et al. 2009; Belz et al. 2016; da Silva Bertão et al. 2021
HPP Salto Osório, Iguaçu river, PR	-25.537985	-53.008446	hydroelectric power plant	Belz et al. 2016
HPP Salto Santiago, PR	-25.628405	-52.613305	hydroelectric power plant	Belz et al. 2016
HPP Gov. Ney Braga de Barros (Segredo), PR	-25.794127	-52.114842	hydroelectric power plant	Belz et al. 2016

Table 2. Comparison among specimens of *Cordylophora* sampled in this study [mean±standard error (range)] and descriptions from the literature. Number in brackets indicates the total number of specimens examined. The symbol "-" indicates lack of the structure to be measured (e.g., gonothecae, retracted tentacles) or lack of information from the literature. HPP Funil = hydroelectric power plant Engenheiro José Mendes Júnior, Minas Gerais state, Brazil; HPP São Simão= hydroelectric power plant São Simão, Goiás state, Brazil.

Measures (μm)	HPP Funil	HPP São Simão	Grohmann (2008)	Folino-Rorem and Renken (2018) Clade 1A	Folino-Rorem and Renken (2018) Clade 2B	Schuchert (2004)	*C. solangiae* (Redier 1967)
Colony							
Total Length (mm)	14.6±2.99 (6-22) [5]	19±2.45 (12-25) [5]	19.05	-	-	up to 30	12-20
Stem							
Diameter	150.73±11.64 (127.36-193.69) [5]	185.50±7.12 (168.86-208.87) [5]	-	-	-	200	215±20.62 (170-250) [4]
Number of annulations	10.40±1.44 (6-14) [5]	11±1.79 (7-17) [5]	-	-	-	-	-
Hydranth							
Length	446.54±28.92 (360.29-494.47) [5]	526.29±50.37 (397.30-673.76) [5]	-	647±51.3	702±24.4	1000-2000	1737.50±189.71 (1200-2050) [4]

Maximum Diameter	390.97±34.14 (272.86-484) [5]	382.96±28.87 (319.26-489.69) [5]	-	322±19.3	326±9.33	-	-
Pedicels							
Length	1241.96±270.94 (547.74-1948.47) [5]	919.96±104.32 (656.66-1214) [5]	-	-	-	-	-
Diameter	126±5.14 (107.18-134.30) [5]	138.76±3.65 (124.51-145.04) [5]	-	-	-	-	-
Number of annulations	5.40±0.24 (5-6) [5]	5.20±0.49 (4-7) [5]	-	-	-	-	-
Tentacles							
Number	13.40±0.98 (12-17) [5]	17±0.45 (16-18) [5]	16-20	14.2±0.75	21.3±0.45	14-16 (up to 27)	50-55
Length	-	-	-	718±95.5	1150±58.9	up to 1400	1925±444.18 (1100-3000) [4]
Male Gonophores							
Length	459.66±78.84 (312.48-668.02) [4]	-	-	-	-	-	622.50±58.79 (450-710)
Maximum Diameter	373.36±60.56 (264.48-538.34) [4]	-	-	-	-	-	425±31.22 (350-490)

Female Gonophores							
Length	-	779.83±28.56 (744.40-843.83) [5]	-	-	-	700-1000	-
Maximum Diameter	-	563.64±32.68 (460.13-653.37) [5]	-	-	-	about half the length	-
Number of oocytes	-	7.60±0.93 (6-11) [5]	-	-	-	7-16	-
Diameter of oocytes	-	143.29±6.34 (126.31-159.34) [5]	-	-	-	70-120	-
Gonophore Pedicels							
Length	94.79±11.44 (82.06-117.63) [3]	141.51±12.14 (110.61-173.53) [5]	-	-	-	-	-
Diameter	116.69±12.85 (91.91-134.98) [3]	134.51±17.37 (97.99-190.46) [5]	-	-	-	-	-
Habitat	Freshwater	Freshwater	Freshwater	Freshwater	Brackish	Brackish	Marine

Table 3. Morphometric characters of the cnidome of specimens of *Cordylophora* from Brazil [mean±standard error (range)] and descriptions from the literature. Number in brackets indicates total number of capsules (or populations*) examined. The symbol "-" indicates lack of information from the literature. HPP Funil = hydroelectric power plant Engenheiro José Mendes Júnior, Minas Gerais state, Brazil; HPP São Simão= hydroelectric power plant São Simão, Goiás state, Brazil.

Measures (µm)	HPP Funil [20]	HPP São Simão [20]	*Cordylophora* sp. Clade 1A* [8]	*Cordylophora* sp. Clade 1B* [4]	*Cordylophora* sp. Clade 2B* [2]	Galea (2007)	Itô (1951) *C. japonica*
Desmonemes							
Length	3.93±0.09 (3.40-4.69)	4.79±0.07 (4.12-5.21)	4.43±0.07 (4.14-4.62)	4.37±0.06 (4.25-4.51)	4.52±0.10 (4.42-4.61)	5.3-5.6	4.9-5.0
Width	2.80±0.05 (2.20-3.11)	3.23±0.05 (2.94-3.58)	-	-	-	2.8-3.2	-
Microbasic Euryteles							
Length	7.23±0.10 (6.21-7.78)	7.31±0.09 (6.60-8.13)	7.91±0.10 (7.56-8.44)	7.93±0.09 (7.73-8.07)	8.20±0.16 (8.04-8.35)	10.2-11.3	10-10.2
Width	4.05±0.07 (3.30-4.48)	4.04±0.08 (3.60-4.7)	-	-	-	4.2-4.9	-

*Folino-Rorem et al. (2009) and Wollschlager et al. (2013)

Table 4. Intra and inter-clade uncorrected p-distances (%) for Cytochrome Oxidase I (COI) sequences of populations of *Cordylophora* analyzed in this study. Minimum, maximum and mean distances are provided.

COI	Min	Max	Mean
Intra-clade			
1	0	8.72	2.44
2	0	15.04	8.98
1A	0	0.68	0.12
1B	0.34	1.71	1.25
2A	0	6.32	2.53
2B	0.17	0.34	0.23
2C	0	0	0
Inter-clade			
1 x 2	12.65	15.90	14.57
2A x 3	14.7	15.04	14.77
2B x 3	14.36	14.53	14.47
1 x 3	14.53	15.9	15.56
1A x 1B	7.86	8.72	8.42
2A x 2B	9.74	10.09	9.94

SUPPLEMENTARY TABLES

Table S1. *Cordylophora caspia* haplotypes referenced in GenBank, with their respective distribution, including sequences from this study.

Species	**Country**	**Locality**	**Latitude (decimal)**	**Longitude (decimal)**	**GenBank**	**Locality code**	**Salinity (< ou = 0.5 PSU: freshwater; >0.5 PSU, brackish)**	**Haplotype**	**References**
Cordylophora caspia	USA	Lake Powell, UT, USA (Lake Powell 1)	36.9375	-111.4837	KU695587.1	LP1_WUSA	Colorado river (freshwater)	H1	Pucherelli et al. 2016
Cordylophora caspia	USA	Lake Powell, UT, USA (Lake Powell 2)	36.9375	-111.4837	KU695588.1	LP2_WUSA	Colorado river (freshwater)	H1	Pucherelli et al. 2016
Cordylophora caspia	USA	Lake Powell, UT, USA (Lake Powell 3)	36.9375	-111.4837	KU695589.1	LP3_WUSA	Colorado river (freshwater)	H1	Pucherelli et al. 2016
Cordylophora caspia	USA	Lake Mead, NV/AZ, USA (Lake Mead 1)	36.0163	-114.7372	KU695590.1	LM1_WUSA	Colorado river (freshwater)	H1	Pucherelli et al. 2016
Cordylophora caspia	USA	Lake Mead, NV/AZ, USA (Lake Mead 2)	36.0163	-114.7372	KU695591.1	LM2_WUSA	Colorado river (freshwater)	H1	Pucherelli et al. 2016
Cordylophora caspia	USA	Lake Mead, NV/AZ, USA (Lake Mead 3)	36.0163	-114.7372	KU695592.1	LM3_WUSA	Colorado river (freshwater)	H1	Pucherelli et al. 2016
Cordylophora caspia	USA	Lake Mohave, AZ/CA, USA (Lake Mohave 1)	35.1967	-114.5683	KU695593.1	LH1_WUSA	Colorado river (freshwater)	H1	Pucherelli et al. 2016
Cordylophora caspia	USA	Lake Mohave, AZ/CA, USA (Lake Mohave 2)	35.1967	-114.5683	KU695594.1	LH2_WUSA	Colorado river (freshwater)	H1	Pucherelli et al. 2016
Cordylophora caspia	USA	Lake Mohave, AZ/CA, USA (Lake Mohave 3)	35.1967	-114.5683	KU695595.1	LH3_WUSA	Colorado river (freshwater)	H1	Pucherelli et al. 2016
Cordylophora caspia	USA	Lake Erie (north side of Gibraltar Island), OH, USA	41.658382	-82.821021	KC489509.1	LE_EUSA	0 (freshwater)	H5	Wollschlager et al. 2013
Cordylophora caspia	USA	Columbia River, OR, USA	46.263611	-124.082497	EF540779.1	CR_WUSA	0 (freshwater)	H6	Folino-Rorem et al. 2009
Cordylophora caspia	USA	Coos Bay, OR, USA	46.429739	-124.229858	EF540780.1	CB_WUSA	11 (brackish)	H7	Folino-Rorem et al. 2009
Cordylophora caspia	USA	James River, VA, USA	37.206369	-76.737683	EF540793.1	V_EUSA	0.5 (freshwater)	H1	Folino-Rorem et al. 2009; Wollschlager et al. 2013

Cordylophora caspia	USA	Illinois River, Henry, IL, USA	41.110225	-89.351117	EF540785.1	H_EUSA	0 (freshwater; Wollschlager et al., 2013); 1 (but labeled as "freshwater" in Folino-Rorem et al., 2009; see Table 1, Fig. 2)	H1	Folino-Rorem et al. 2009; Wollschlager et al. 2013
Cordylophora caspia	USA	DesPlaines River, IL, USA	41.525194	-88.086425	EF540781.1	DP_EUSA	0-0.5 (freshwater)	H1	Folino-Rorem et al. 2009; Wollschlager et al. 2013
Cordylophora caspia	USA	LaSalle River, IL, USA	41.294289	-88.628081	EF540789.1	LS_EUSA	0 (freshwater)	H3	Folino-Rorem et al. 2009; Wollschlager et al. 2013
Cordylophora caspia	USA	Lake Michigan, IL, USA (Burnham Harbor, Chicago, IL, USA	41.853436	-87.610375	EF540788.1	BH_EUSA	0.1 (freshwater)	H3	Folino-Rorem et al. 2009
Cordylophora caspia	USA	Squamscott River, NH, USA	42.983061	-70.948508	EF540782.1	E_EUSA	8-10 (brackish)	H2	Folino-Rorem et al. 2009; Wollschlager et al. 2013
Cordylophora caspia	USA	Woods Hole, MA, USA	41.527072	-70.669644	EF540794.1	WH_EUSA	3-15 (brackish)	H8	Folino-Rorem et al. 2009; Wollschlager et al. 2013
Cordylophora caspia	USA	Napa River, CA, USA	38.197186	-122.315997	EF540790.1	NR_WUSA	16 (brackish)	H9	Folino-Rorem et al. 2009; Wollschlager et al. 2013
Cordylophora caspia	USA	Petaluma River, CA, USA	38.167078	-122.538858	EF540792.1	PR_WUSA	22 (brackish)	H10	Folino-Rorem et al. 2009
Cordylophora caspia	Panama	Panama Canal, Gamboa, Panama	9.115239	-79.705556	EF540791.1	P_Panama	0 (freshwater)	H1	Folino-Rorem et al. 2009
Cordylophora caspia	Chile	Huinay, Chile	-41.477428	-72.931839	EF540778.1	C_Chile	Brackish	H11	Folino-Rorem et al. 2009
Cordylophora caspia	France	Canet-Saint-Nazaire, France	42.674236	3.000228	EF540783.1	F_France	Brackish	H2	Folino-Rorem et al. 2009
Cordylophora	Hungary	Lake Balaton, Tihany,	46.914439	17.888183	EF540787.1	LB_Hungary	0.45 (freshwater, but labeled as "brackish"	H12	Folino-Rorem et

caspia		Hungary					in Folino-Rorem et al., 2009; see Table 1, Fig. 2)		al. 2009
Cordylophora caspia	Germany	Ryck River, Germany	54.099717	13.387606	EF540784.1	G_Germany	5 (brackish)	H2	Folino-Rorem et al. 2009
Cordylophora caspia	Ireland	Shannon River, Ireland	52.665647	-8.630678	EF540786.1	I_Ireland	2 (brackish)	H2	Folino-Rorem et al. 2009
Cordylophora caspia	Brazil	UHE São Simão, Goiás (SPIC), Rio Paranaíba, Brazil	-19.018683	-50.500142	ON568498	GO_Brazil	Paranaíba river (freshwater)	H4	This study
Cordylophora caspia	Brazil	UHE Engenheiro José Mendes Júnior (Funil), Rio Grande, Minas Gerais, Brazil	-21.143139	-45.036194	ON568497	MG_Brazil	Rio Grande river (freshwater)	H4	This study

5. Capítulo 3.1

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REVIEW PAPER

Genetic and functional repertoires of *Limnoperna fortunei* (Dunker, 1857) (Mollusca, Mytilidae): a review on the use of molecular techniques for the detection and control of the golden mussel

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Abstract *Limnoperna fortunei*, the golden mussel, is a bivalve mollusc native to Southeast Asia, which was introduced to South America via ballast water from commercial cargo ships from China and South Korea. In Brazil, the golden mussel is considered an invasive species, which has caused enormous economic and environmental damage. This is mainly due to the obstruction of pipes carrying water for the cooling systems within hydroelectric power plants. The presence of this invasive species also negatively impacts native fauna and flora. Early detection and rapid response during the early stages of infestation for this invasive species is essential to effectively control populations and prevent new infestations from forming. Techniques within molecular biology allow for more efficient detection of the golden mussel in all stages of development. In addition, the analysis of the genome, transcriptome and proteome of this species makes it possible to identify targets of biotechnological interest, which may be useful for the control of the golden mussel or for the generation of products of technological innovation. This review presents an overview of molecular studies involving this species and the perspectives on the prevention, control and combat of the golden mussel using these approaches.

Keywords Invasive species · *Limnoperna fortunei* · Molecular biology · Genome · Transcriptome

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Introduction

Limnoperna fortunei (Dunker, 1857), popularly known as the golden mussel, is a bivalve mollusc belonging to the Mytilidae Family (subclass Pteriomorphia and order Mytiloida) and native to Southeast Asia (including China and South Korea). From the 1990s, however, the golden mussel was introduced in the South American countries, possibly via ballast water from ships of commercial routes (Pastorino

et al., 1993). The golden mussel was initially introduced into the estuary of the La Plata River in Argentina (1991) and spread over 5,000 km along the coasts of Uruguay, Brazil, Paraguay, and Bolivia (Darrigran & Pastorino, 2004). Recent reports registered the presence of the species in the São Francisco River Basin, in the transposition canals of the Sobradinho Hydroelectric Plant, located on the border between the states of Bahia and Pernambuco (Brazil) (Barbosa et al., 2016).

Several characteristics of the golden mussel favour their success as an invasive species. Among them, the presence of two shells made of calcium carbonate, which provide an ecological advantage by protecting them from external factors, such as temperature variation, salinity, pH and predation (Morton, 1977; Darrigran, 2002; Uliano-Silva et al., 2016). Further, *Limnoperna fortunei* present phenotypic plasticity which favours their adaptation to changes in the environment (Uliano-Silva et al., 2015). They are gonochoric, with external fertilisation and indirect development, conferring great reproductive success (Morton, 1977). The larvae are one of the main forms of dispersion and the high success rate of the species is linked to the rapid reproduction of individuals at the onset of invasion, as well as the lack of specialied predators and parasites within the invaded environments (Darrigran & Mansur, 2006). In South America, reproduction is estimated to occur over at least 9 months per year, being proportionally more intense in periods of higher water temperature. *Limnoperna fortunei* individuals reach sexual maturity after approximately 1 year (Morton, 1982; Uliano-Silva et al., 2015).

The golden mussel has caused several environmental and economic impacts for Brazil and other South American countries. Among the environmental impacts, its occurrence has encouraged blooms of phytoplankton and toxic cyanobacteria (Boltovskoy et al., 2009; Gazulha et al., 2012; Frau et al., 2013). Occurrences of *L. fortunei* further result in reduced turbidity and organic matter, and increased concentrations of dissolved ammonia, nitrates and phosphates (Boltovskoy et al., 2009). Economically, they have brought losses to the country's hydroelectric power plants by obstructing the pipelines of water abstraction systems, resulting in high annual operating and maintenance costs (Nakano & Strayer, 2014). This obstruction from the fouling is related to the production of byssus threads, which are secreted by glands located in the foot of these bivalve organisms. The adhesion between the byssus and the substrate occurs on a nanoscale, through capillary interactions and van der Waals forces (Meyers et al., 2008; Andrade et al., 2015), allowing the golden mussel to attach to almost any substrate such as rocks, stems, shells from other molluscs, the underside of boats, fishing nets, glass and Teflon plates (Faria et al., 2006). To get an idea, in the United States, losses caused by invasive species are estimated at 143 billion dollars a year. In Brazil, Cemig, one of the main groups within the electrical energy sector has invested more than R$ 10 million in R&D projects to mitigate the invasion problem (Silva et al., 2016).

There is still no standard methodology for controlling the golden mussel which is efficient for the different environments it invades. Chlorine is an alternative for the control of this mollusc in the industry, but it is dangerous for the environment (Rajagopal et al., 1991). Some chemicals have also been used, but these were not successful due to the mussels closing their shells in the presence of toxic compounds in the water. In order to circumvent this limitation, Calazans et al. (2013) reported which the microencapsulation of biocides might be an efficient measure for the control of the golden mussel.

Just as it is important to develop control measures for the golden mussel in a particular environment, it is also important which it can be detected early in its dispersal. Early detection and rapid response at the onset of the invasive process is essential to control the growth of subpopulations, preventing further infestations from forming in new environments (Ziller et al., 2007; Vander Zanden & Olden, 2008). However, it is difficult to identify the larvae of bivalve molluscs. Optical identification techniques do not work with most of these larvae, since species-specific characteristics only appear in the later stages of development or are visible only at the microscopic level (Garland & Zimmer, 2002). Also, in some cases, morphological features intuitively used for diagnosis in adult individuals, such as shell colour, shape and structure variations, and

scars of muscle insertions, may be influenced by environmental factors (Pie et al., 2006).

Significant tools within molecular biology have been developed for the study of this invasive species, such as the development of molecular markers which allowed the precise identification of individuals or groups at any stage of development and in addition made possible the resolution of systematic questions (Rees et al., 2014). Nucleotide and protein sequences generated by the "omics" technologies allowed searching for possible new targets within the genetic repertoire for different purposes. For example, the comparison between the transcriptomes of the blue mussel invasive species *Mytilus galloprovincialis* Lamarck, 1819 with the native congener *Mytilus trossulus* Gould, 1850 allowed elucidating the molecular mechanisms of adaptation and tolerance to environmental shifts such as temperature (Lockwood et al., 2010) and salinity (Lockwood & Somero, 2011), reveal new molecular targets to the control of this invasive species. In addition, the comparison of the proteomes of these two blue mussel species after acute heat stress showed differences in chaperoning, proteolysis, energy metabolism, oxidative damage, deacetylation and cytoskeleton composition, revealing the mechanisms which allow the expansion of this invasive species in warm waters (Tomanek & Zuzow, 2010; Fields et al., 2012). Proteomics data could show similar differences between the mechanisms of temperature acclimatisation used by the snails *Pomacea canaliculata* (Lamarck, 1822) and *Pomacea diffusa* Blume, 1957 (Mu et al., 2015). Molecular studies focusing on *L. fortunei* have been conducted since 2006 (Fig. 1), but still lack further bioinformatics analysis of their genetic repertoire and related functions. These data are needed to expand knowledge about invasive genetic traits and to develop efficient biotechnology strategies for the control of the golden mussel (Uliano-Silva et al., 2015).

In this review, we compiled studies which used molecular approaches to discover the genetic and functional characteristics of *L. fortunei*, aiming to provide an overview of what has been done and what can be further explored. Thus, it will be possible to identify molecular targets with biotechnological potential, as well as to select the most promising approach which will help in the prevention and control of the golden mussel as an invasive species.

Molecular approaches for the detection and control of the golden mussel

Techniques for rapid detection of the golden mussel

According to the discussion in the European Commission (2013),[1] it was suggested which initial efforts should focus on the early detection and rapid response to invasive species. For the results obtained in the research to feasibly be used in the field, it is essential which the methods can be applied on a large scale and are economically accessible, with molecular markers being important tools for this purpose.

Hebert et al. (2003) proposed the use of a small sequence ($\sim$ 650 bp) of mitochondrial DNA, which codes for the subunit I of Cytochrome C Oxidase (COI), which, due to its variability, can be efficiently used to establish intraspecific and interspecific relations. The method was termed as DNA barcoding. In 2006, Pie et al. tested the specificity of primers for the amplification of *L. fortunei* COI by PCR. The tests compared DNA samples from different molluscs, including other invasive species such as *Corbicula fluminea* (Müller, 1774). Fragments of COI could be amplified only in *L. fortunei* samples, even in small concentrations of DNA. Thus, confirming the specificity and sensitivity of this set of primers for the detection of the golden mussel.

Subsequently, Boeger et al. (2007) used these primers to test the efficiency of detection in plankton samples containing larvae of the golden mussel and compared this approach with alternative sampling methods such as microscopy and manual searching for adult individuals. The results of this study demonstrated which the molecular method is more advantageous and essential for the large-scale monitoring of the golden mussel in invaded environments. The group attested the sensitivity of PCR in samples with known amounts of larvae (1–5 larvae in 2,000 m^3 of filtered water). This work also tested different adjuvants to neutralise the PCR inhibitors found in environmental samples. All three added adjuvants (BSA—bovine serum albumin, DMSO-dimethylsulfoxide and

[1] Proposal for a regulation of the European Parliament and of the Council on the prevention and management of the introduction and spread of invasive alien species. http://ec.europa.eu/environment/nature/invasivealien/index_en.html.

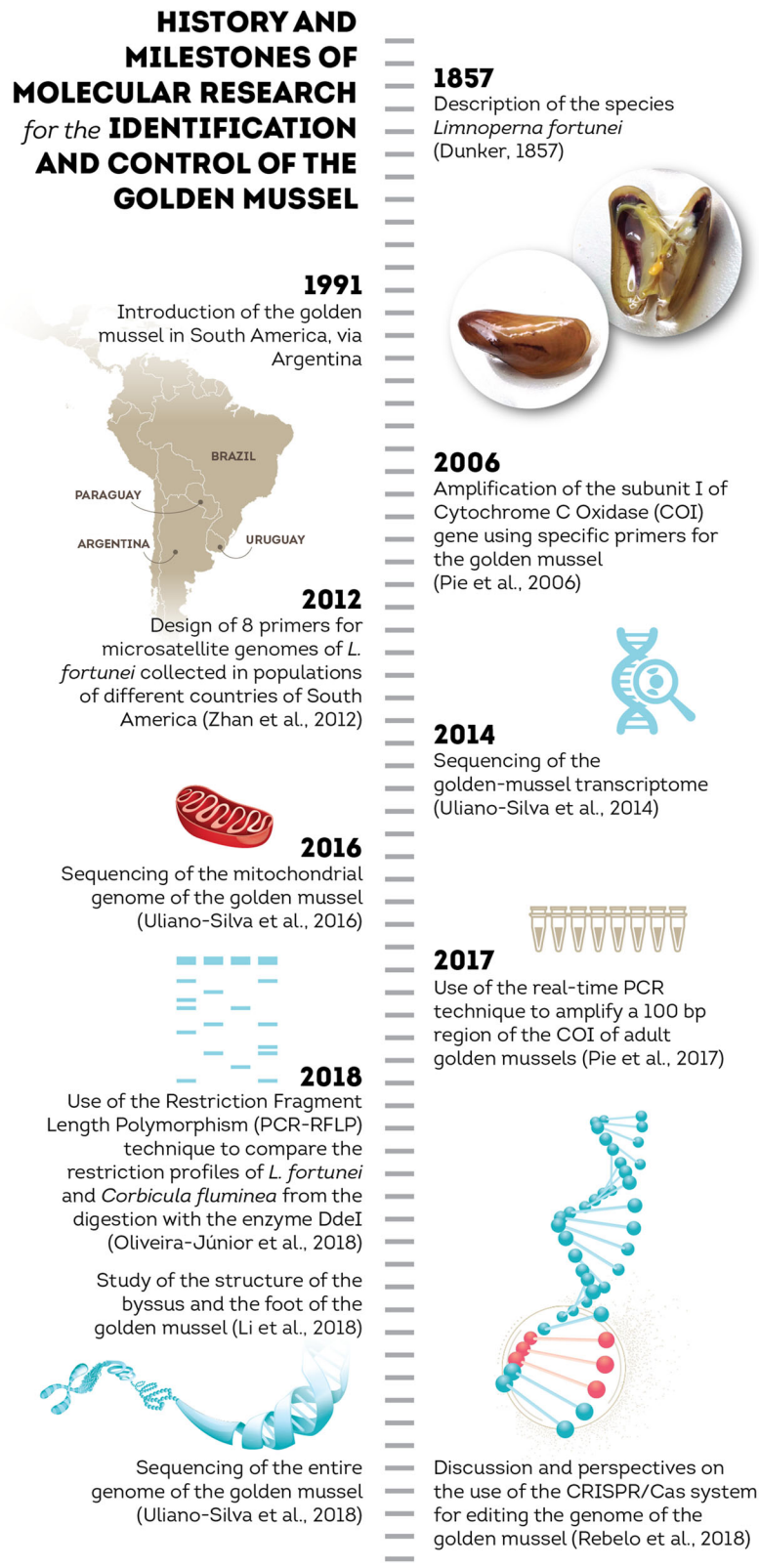

Fig. 1 History and milestones of molecular research for the identification and control of the golden mussel

glycerol) were shown to be efficient in preventing inhibition of the reaction, particularly the BSA. Pie et al. (2017) used the real-time PCR technique (more sensitive than conventional PCR) to amplify a section of 100 bp from the COI of adult golden mussels. The technique was able to detect 0.225 pg of the target DNA, evidencing its extreme sensitivity.

Other methods have also been tested to corroborate and give robustness to the identification of the species and to avoid the emission of reports which do not confer with the reality of an environment. Oliveira-Junior et al. (2018) utilised the PCR–RFLP technique (Restriction Fragment Length Polymorphism) to generate different restriction patterns between *L. fortunei* and *C. fluminea*, whose larvae and adults can coexist in the same environment (Karatayev et al., 2015). DNA digestion with *DdeI* restriction enzyme allowed the identification of a 100 bp fragment in *L. fortunei* which was absent in *C. fluminea* (Karatayev et al., 2015). Thus, this simple and economically more viable method allowed to discriminate between the larvae of these two species in planktonic samples, solving the problems of conflicts regarding the morphological identification of these species.

Genetic diversity of golden mussel populations

The adaptation of a population to a new environment and its dissemination are important steps for the successful establishment of invasive species (Daehler, 2003; Blackburn et al., 2011). Phenomena such as phenotypic plasticity, genetic variability and adaptive homeostasis are mainly responsible for the ability of a species to adapt quickly to spatial or temporal environmental variations, thus ensuring an advantage in an invaded environment (Wells & Pigliucci, 2000; Ghalambor et al., 2007; Hulme, 2008). The distribution and genetic structure of the introduced populations may exhibit complex profiles. A modern view of the biology of invasions recognises which a high genetic diversity is not a rule among the populations introduced. Some studies have documented invasive populations with similar genetic diversity; however, in the case of multiple invasions within a single environment higher genetic diversity was seen (Taylor & Keller, 2007; Handley et al., 2011). New genotypes may appear in invasive populations due to the hybridisation of different lineages which originated in source populations or hybridisation with native species (Keller & Taylor, 2008).

The COI gene was also used to identify haplotypes in *L. fortunei* to form the genetic profile of populations in their process of expansion in territories of various parts of the globe. A total of 20 haplotypes were identified in 26 populations of golden mussels distributed throughout Japan (Tominaga et al., 2009). These data were analysed by phylogenetic methods which revealed the relation of ancestry between these populations and allowed for the prediction of possible routes of invasion for each population. In another study, the use of the COI gene and eight microsatellites revealed which the genetic patterns of 22 mussel populations scattered throughout South America varied geographically; and which the "leap" dispersion dynamics occur as a function of vessel movement across the continent (Zhan et al., 2012).

Although there were morphological differences between the 24 golden mussel populations scattered across the La Plata River in Argentina, analysis of the COI gene did not identify any quantitative differences in haplotype or nucleotide diversity (Paolucci et al., 2014). These data suggest which the populations of mussels introduced in South America show a pronounced morphological variation, especially in the measurements of shells and gills, which seem to result from developmental plasticity, as has been observed in other mussel species (Peyer et al., 2010). These adaptations may have facilitated the spread of this species to a wide range of habitats.

Zhan et al. (2012) designed eight microsatellite primers of the *L. fortunei* genome collected from populations of different South American countries. However, a study by Furlan-Murari (2016), demonstrated difficulties in using these same primers in populations of the Paranapanema River in Paraná, Brazil. This difficulty revealed the need for the development of new markers which could identify a greater number of individuals and thus reach the high genetic variability of the population. Thus, de Souza et al. (2018), using *L. fortunei* enriched genomic libraries, identified and characterised 13 more microsatellite markers, eight of which were polymorphic.

In addition to these regional studies on the genetic diversity of the golden mussel, Ghabooli et al. (2013) analysed the COI gene and eight microsatellites of individuals of this species in different localities. It was

revealed which native mussels from Asia present a higher genetic diversity than those from South America. This difference in genetic diversity is probably due to the transfer of propagules resulting from intense navigational activity between different Asian territories.

Furlan-Murari et al. (2019) evaluated the genetic diversity of *L. fortunei* in fish farms of 3 reservoirs belonging to the Paranapanema River, in the state of Paraná, Brazil. The targets of this analysis were eight microsatellites (Zhan et al., 2012), 5 of which were successfully amplified (Lf06, Lf07, Lf21, Lf22, and Lf23), resulting in 38 alleles. The results showed lower than expected heterozygosity for all populations; in addition to intra-populational genetic variability greater than inter-populational, suggesting a genetically diverse founding population. It was also observed the occurrence of gene flow in all populations, along with the absence of a recent bottleneck effect. The data from this study indicated a possible dispersion of *L. fortunei* upstream, likely caused by anthropogenic factors.

The genome of the golden mussel

Genomic analysis is an important tool for understanding genetic mechanisms related to the process of colonisation and adaptation of invasive species and their ability to cope with rapid environmental changes (Rius et al., 2015). For example, Phuc et al. (2007) genetically engineered *Aedes aegypti* (Linnaeus, 1762) male mosquitoes (strain OX513A) expressing a conditional lethality trait and a fluorescent marker. When released into the environment, male OX513A mosquitoes mate with wild females. Their offspring inherit the additional genes and die before reaching adulthood, leading to a reduction in the population of wild *A. aegypti* mosquitoes (Phuc et al., 2007).

The mitochondrial genome of the golden mussel was recently obtained using the Illumina HiScanSQ sequencing platform (Uliano-Silva et al., 2016). Among the findings, it was possible to reveal which, as observed in other bivalves, the mitochondrial genome of the golden mussel presents a high genetic variability, proving to be an important tool for phylogenetic studies (Breton et al., 2011; Uliano-Silva et al., 2016). The genome also reveals which *L. fortunei* derives from the oldest common ancestor of members of the Mytilidae family (Uliano-Silva et al., 2016).

The complete genome of *L. fortunei* was also sequenced recently, using the Illumina HiScanSQ, Illumina HiSeq 1500 machine and Pacific Biosciences (Uliano-Silva et al., 2017). The complete prediction of genes is underway and may reveal targets which could be used to control the species, such as the genes encoding the adhesive proteins produced by the glands of the foot (Uliano-Silva et al., 2014); and genes related to the reproductive system of the species, which have been shown to be effective in invertebrate population control (Hammond et al., 2016).

Genomic edition by the CRISPR/Cas9 system and feasibility of its use for the control of the golden mussel

Since 2012, the CRISPR/Cas9 system has aroused the interest of the scientific community for its ability to edit the genome of any species with a known DNA sequence (Gonçalves & Paiva, 2017). This system is currently being used for the study of cancer genetics (Sánchez-Rivera & Jacks, 2015). It has been shown to be a powerful tool for the suppression of hepatitis B virus (Lin et al., 2015) and for modelling of neurological disorders (Heidenreich & Zhang, 2016). The CRISPR/Cas9 system was also used to control two vectors of malaria; firstly in the *Anopheles gambiae* Giles, 1902 which study identified three genes which confer a sterility phenotype on females (Hammond et al., 2016); and secondly in the *Anopheles stephensi* Liston, 1901, which resulted in progeny of males and females derived from transgenic males exhibiting a high frequency of germline gene conversion (Gantz et al., 2015). The versatility and viability of CRISPR-Cas9 system have also been demonstrated in *Crepidula fornicata* (Linnaeus, 1758) and *Lymnaea stagnalis* (Linnaeus, 1758) molluscs (Perry & Henry, 2015; Abe & Kuroda, 2019) mbryos expressing mCherry fused to endogenous β-catenin. This genome editing should permit the creation of stable lines of transgenic snails for breeding purposes. Besides, Abe & Kuroda (2019) firstly demonstrated, using CRISPR/Cas9 system, which the *Lsdia1* gene is responsible for dextral/sinistral coiling at the one-cell stage, highlighting this importance for chiromorphogenesis in invertebrates and vertebrates.

After this study in invertebrates, Rebelo et al. (2018) have been raising the hypothesis of the feasibility of using this system in the control of the golden mussel. However, problems such as obtaining large-scale embryos under laboratory conditions, as well as risks, such as the reintroduction of the genetically modified species in the environment, may still represent major obstacles (Rebelo et al., 2018).

Transcriptome of the golden mussel

Uliano-Silva et al. (2014) sequenced the transcriptome of five tissues of adult *L. fortunei* individuals by pyrosequencing using the Roche FLX 454 platform. Genes involved with filtration rates and production of the byssus; antioxidant enzymes and chaperones, related to the ability to survive in waters with dissolved oxygen variation and temperature are among the mRNAs identified. *L. fortunei* presented an expansion of the *heat shock protein* 70 family (hsp70), with 33 different proteins annotated. These proteins, which are commonly found in other bivalves, are involved in their adaptation to extreme environments (Zhang & Zhang, 2012). However, the absence of genes related to the adaptation to oxidative stress can restrict its occurrence, being a possible target for a molecular approach to the control of the golden mussel.

A recently study published by Afonso et al. (2019) sequenced total RNA samples from male and female golden mussel gonads in the spawning stage. The data obtained revealed 3,906 differentially expressed transcripts between male and female. The differentiation pathways were compared between golden mussel and other bivalves, unveiling 131 potential homologs, of which 15 were overexpressed in males and four in females (Afonso et al., 2019). Just as important as to understand the mechanisms of gene expression in the adult organism, it is necessary to understand the inherent gene expressions during embryonic and larval development, which is still lacking in the *L. fortunei* larval stages.

Proteomic studies of the golden mussel

Li et al. (2018) aimed, unprecedentedly, to understand the structure of the byssus and identify the proteins of the foot and to detect the metal ions of the foot tissue and byssus of the golden mussel by using different approaches including scanning electron microscopy, liquid chromatography/tandem mass spectrometry, transcriptome sequencing, quantitative PCR and inductively coupled plasma mass spectrometry. The data suggested which the structural features of the adhesive plate, proximal and distal, are highly useful for the adhesion of the byssus. In addition, 3,4-dihydroxyphenyl-a-alanine (Dopa) has been shown to be the major post-translational modification of the byssus. They also showed which potential interactions between the 16 proteins (e.g. Lfbp-1, Lffp-2 and Lfbp-3) identified at the foot of the golden mussel and some specific metal ions (e.g. Ca^{2+} and Fe^{3+}) may be important for the structural integrity and adhesive properties of the byssus. Knowledge about the mechanisms of byssus adhesion in freshwater mussels, revealed in this study, is indispensable for the development of strategies against the encrustation of these invaders.

Conclusions and perspectives

In this review, we highlight the research carried out to date, of the molecular approaches used to investigate the genetic repertoire of the golden mussel and related functions. The integrative genetics enable large-scale genome-wide studies and the screening of transcript levels and proteins of one or more species. These approaches provide consistent information to redesigning organisms for different purposes, being this field known by synthetic biology. The genome sequence is one of the most relevant and informative descriptions of the biology of a species, being of prime importance to understand and control biological invasion. By compiling the available data, it was observed which the molecular techniques for the detection of the golden mussel are well established and are efficient to identify this species in different stages of development—from the larval phase to the adult individual. In addition, the molecular markers used to identify the golden mussel have been useful for tracing phylogenetic relationships between the different haplotypes of the invasive populations and for predicting possible invasion routes in different continents.

As for the sequencing of the genome and transcriptome of the golden mussel, studies were conducted resulting in the availability of good quality sequences,

which should continue to be curated, in order to extract as much information as possible and to screen genes and functions of biotechnological interest. Previously, genes related to the mussel's reproductive system and its ability to survive in different environments were detected within these studies. These genes may be important targets for the control of the golden mussel invasion. The proteomic study of the foot and byssus of the golden mussel also revealed which the mechanisms of adhesion of the byssus could be important for the development of measures which prevent the build-up of these organisms in the pipes of the systems of abstraction of water.

Finally, the promising use of the genomic edition by the CRISPR/Cas9 system, in order to modify the golden mussel in favour of its control, can be a viable tool according to scientific and economic study. The 26 genes identified, related to the reproduction of this species (Hammond et al., 2016), should be investigated as possible targets for this technique of genomic editing and to be tested in the future on a large scale.

Although not yet established as a model organism, since the reproduction of *L. fortunei* in laboratory conditions was not feasible, the studies presented in this review (and others to come) serve as a support and platform for creating tools which help in the prevention, control and the combat of new invasions or the mitigation of existing invasions, by focusing on the molecular basis of the golden mussel.

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6. Capítulo 3.2

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A Case for the Continued Study of the Golden Mussel Invasion of Brazil: Efficient Detection and Containment of *Limnoperna fortunei* (Dunker, 1857) Dispersion Involves Multiple Approaches and Different Actors

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Abstract

The establishment of invading organisms in natural ecosystems is one of the most serious environmental issues. In Brazil, the invasive species *Limnoperna fortunei* (Dunker, 1857), the golden mussel, is a mollusk capable of causing major changes in water systems, generating social and economic impacts, given its biofouling capacity. *Limnoperna fortunei* can easily block pipes and heat exchangers in the water systems of hydroelectric power plants due to its ability to strongly adhere to the substrate using its byssus - a bundle of filaments secreted by these animals. Therefore, the early detection of this invader is essential for management actions to be immediate, in order to control population growth rate at the beginning of the invasive process, preventing this environment from serving as a source for new infestations. The implantation of a method that integrates the active monitoring of prioritized areas, laboratory techniques, including molecular biology methods, and the sharing of hydrographic data between basin managers and users for early detection of the presence of species in Brazilian waters appears as an efficient option to prevent and control invasions.

Keywords: *Limnoperna fortunei*; Golden mussel; Invasive species; Early detection; Molecular biology; Biofouling; Economic losses

Introduction

Biological invasions by alien species provoke ecosystem imbalance, thus affecting the anthropic use of natural resources [1]. *Limnoperna fortunei* (Dunker, 1857), popularly known as the golden mussel, is an example of an invasive species. This bivalve mollusk belongs to the Mytilidae Family (Subclass Pteriomorpha, and Order Mytiloida), native to Southeast Asia and was first introduced to South America in 1990, probably via ballast waters from ships on commercial routes [2,3]. Specifically, in Brazil, the golden mussel is established in the South, Southeast, Pantanal biome and, more recently, its presence was reported in the Northeast region, on the border between the states of Bahia and Pernambuco [4,5].

Morphophysiological aspects of the golden mussel guarantee its success as an invasive species: (1) they are protected by two valves (shells), composed of calcium carbonate, which support the soft body and provide protection against external agents [6,7]; (2) they are prolific filter feeders, as the clearance rates for the golden mussel are among the highest reported for suspension feeding bivalves, comparable to other invasive species such as *Dreissena polymorpha* (Pallas, 1771), *Dreissena bugensis* (Andrusov, 1897) *and Corbicula fluminea* (Müller, 1774) [8]; (3) they are gonochoric animals (rare hermaphroditism events were previously confirmed), with external fertilization and indirect development [6,9]. The *L. fortunei* gonads present cyclic maturation stages that

may vary according to the season, and can be divided into four stages: regression, spawning, development, and regeneration. The individual becomes an adult after a period of approximately one year, thus reaching sexual maturity.

However, the spawning phase was observed in the female, only during the summer months, a time when the water temperature reaches its highest point [9]. Due to the large quantity of larvae released into the water they make excellent candidates for promoting new invasions, not only because of their large numbers but also because of their microscopic size [10]. Several countermeasures and anti-fouling treatments have been proposed to mitigate *L. fortunei* macrofouling observed in industrial plants [11-13]. The physical (eg, backwashing) and chemical (e.g., tannins and chlorine) antifouling methods are the most used, however, they still lack specific regulation by the Brazilian environmental agencies, besides the high implantation costs and uncertainty on their efficiency and the impact of their prolonged use [14]. Thus, the early detection of *L. fortunei* in the environment is essential to control the spread while the populations are still small enough to be eradicated, minimizing the chances of success of the invasion [15].

In addition, early detection allows the establishment of sanitary barriers to prevent the invasion of new environments. However, larval identification of *L. fortunei* by microscopic evaluation can be difficult since species-specific morphological characters only appear in the latest stages of the development [16]. Though some of the developmental stages of the larvae can also resemble those of other mollusk species which may lead to inconclusive results in some cases. Molecular biology emerges as a powerful tool that assists in early detection, species identification, and the monitoring of invaded and non-invaded areas. Since 2006, when Pie et al. designed the primer set for amplification of *Cytochrome C Oxidase Subunit I* (*COI*) gene for *L. fortunei* DNA barcoding, much progress has been made towards molecular approaches targeting the golden mussel [17].

Including population variability studies, establishment of advanced techniques for detecting and releasing golden mussel 'omics' (genomes, transcriptomes and proteomics), and the perspective of genetic editing with the CRISPR/Cas9 system. The compendium of molecular information from *L. fortunei* was assembled in the review published by de Paula et al. (2020), which presents a concise timeline highlighting the state-of-the-art research regarding the molecular aspects of *L. fortunei* [18]. Recently, Ludiwig et al. (2021) evaluated the haplotype network of the Brazilian golden mussel populations, focusing on the population of the Northeastern of Brazil, in an attempt to outline the possible invasion routes used by *L. fortunei*. This review of the possible invasion routes has emphasized the need for the creation of stricter sanitary barriers. The main concern of these latter authors is the risk of arrival of the golden mussel in the Amazon basin [19].

The presence of the golden mussel in Brazil and other South American countries has been linked to several economic and environmental impacts. Among the environmental impacts, the presence of *L. fortunei* is related to processes such as cyanobacterial blooms, nutrient recycling, and increased transparency of the water column [1]. Economically, the macrofouling resulting from the establishment of the golden mussel in water cooling and abstraction pipes and systems in hydroelectric plants generates high maintenance and operation costs [20]. This fouling ability is related to the production of byssus threads, a bundle of filaments secreted by glands located on the foot of *L. fortunei*. The formation of byssus is extremely organized, synchronized, complex and rapid, involving the presence of vesicles containing collagen in the liquid crystal phase, which seem to be used in the assembly of these filaments [21].

Despite the damage resulting from the *L. fortunei* invasion, the biological study and monitoring of such invasions brings many benefits. In fact, researchers, institutes, and industries are increasingly interested in discovering and developing new technologies, patents and products based on the careful observation of the biofouling process, such as the synthesis of purified bioadhesives from mussels and other organisms [22,23].

Conclusion

Can the answers to biological invasions be found within the invasions themselves? Are we able to gain enough knowledge from studying these invaders to begin to see the benefits as well as the consequences of their invasions? It may be impossible to find the answers to the questions these invaders pose without the right funding, resources, and technologies. By recognizing that this is a problem worthy of study and resources we have already taken the first step in solving it. First, we must characterize the ecological and biological aspects of the golden mussel only then can we propose antifouling strategies which are specific in their abilities to combat this invasion process whilst maintaining the ecosystem balance within these environments.

Over the years, much progress has been made, in different frontiers of knowledge, with regard to the characterization of the mechanisms involving *L. fortunei*. Many hydroelectric plants, the main industrial sector which suffers directly from the invasion of the golden mussel, have adapted to the presence of this mollusk in their facilities. They have been forced to create alternatives to establish minimally and economically viable coexistence strategies. Regardless of the approach used, many of the proposed solutions were achieved due to the effort of researchers who persist in understanding the phenomena that revolve around the invasion of *L. fortunei*. However, there is an important caveat here: the discord between the timelines of scientific discovery which oftentimes moves at a slow and steady pace, and the urgency with which these solutions need to be implemented to combat the problems industries are facing on a daily basis can often result in

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tension between two groups who are working towards the same goal.

After a large volume of data, information and responses are obtained, it is necessary to make certain that this knowledge is not restricted to researchers and industrial managers but be disseminated to society at large. This information is especially impactful in the hands of those directly involved with aquaculture, such as fishermen's associations, small and medium-sized farmers and even tourist activities, which for lack of information, can transport the golden mussel to an area that has not yet been invaded. Thus, it is important that incentives for studies on the biology of invasions include environmental education and scientific divulgation activities which may reach a wider proportion of those coming into contact with the golden mussel, oftentimes without realizing.

The fact is when it comes to biological invasions prevention is better than cure. Whilst these invaders can promote the creation of new areas of study and new technologies the economic impacts, they can generate can be incredibly detrimental to communities facing them. The mobilization and formation of work groups and task forces, which have diverse profiles and professionals, and integrate different areas of knowledge in multidisciplinary approach (currently, in addition to the classical areas, there is a movement of areas involving computing, engineering, mathematics and robotics, corroborating the mitigation of macrofouling), become essential for decision-making in the face of biological invasion. Therefore, it is important to continuously invest and promote research that increases the understanding of the morphophysiological, genetic, developmental, and ecological aspects of *L. fortunei*, in order to obtain a holistic view of the invasion process caused by this mollusk.

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7. Capítulo 3.3

Standardization of Loop-mediated Isothermal Amplification (LAMP) assay for *Limnoperna fortunei* (Dunker 1857) detection

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Abstract

Among the invasive species that populate South America, the bivalve mollusc *Limnoperna fortunei*, popularly called the golden mussel, stands out. The presence of *L. fortunei* is linked to several environmental and economic issues, being a mitigation action to detect this invader as soon as it reaches an environment not yet invaded. Over the years, molecular methods PCR-based have become more common for detection of *L. fortunei*. However, all these methods are linked to complex logistics from field sampling to laboratory processing. In this case, the use of methods that can be applied directly in the field can speed up the detection process. The aim of this work was to stablish, for the first time, the loop-mediated

isothermal amplification (LAMP) method for the detection of *L. fortunei*, with perspectives for *in situ* application. A set of primers designed for LAMP was tested for DNA amplification from *L. fortunei* adult tissues and environmental samples containing bivalve larvae. Sensitivity tests showed that the limit of detection was 0.01 ng of DNA from tissue samples from *L. fortunei* adults and a minimum reaction time of 60 minutes was required, a considerable time to streamline current detection processes. The set of primers used proved to be species-specific for *L. fortunei*, since there was no amplification of other bivalve or invasive molluscs that co-occur with the golden mussel in the same environment. LAMP also proved to be efficient in the amplification of DNA from samples containing bivalve larvae, demonstrating a resistant method to the inhibition of the reaction against intrinsic characteristics of the environment. Although the results obtained here have been performed in a controlled laboratory environment, the LAMP method seems to be an important tool to integrate *L. fortunei* invasion monitoring protocols, emphasizing the need of future studies focusing on improvements to make this method viable for field-based approaches.

Keywords: *Limnoperna fortunei*, golden mussel, isothermal amplification, LAMP, early detection.

1 Introduction

Limnoperna fortunei (Dunker 1857) is a freshwater bivalve mollusc commonly known as the golden mussel. Originally from Southeast Asia, *L. fortunei* is considered an invasive species that spread across South America, being introduced to this continent possibly via ballast water from ships as a result of international trade (Pastorio et al., 1993). The golden mussel was first introduced to the Rio de la Plata estuary in Argentina in 1991, and one of the last reports of *L. fortunei* occurred in Brazil, in the transposition channels of Rio São Francisco, at the Sobradinho hydroelectric power plant, located on the border

between the states of Bahia and Pernambuco (Barbosa et al. 2016).

The presence of the golden mussel has been associated with several economic and environmental impacts. Economically, the macrofouling caused by *L. fortunei* clogs cooling water pipes in hydroelectric power plants, generating high maintenance and operation costs (Darrigran et al., 2007; Darrigran, & Damborenea, 201; Nakano et al., 2014). In a recent survey, among the 16 relevant invasive species that generate the most cost for Brazil, the only aquatic one was *L. fortunei*, whose expenses are estimated at approximately 10 million dollars per year. This fact allocated the golden mussel in social and ecological activities, with emphasis on control, damage repair, prevention and research (Adelino et al., 2021). Among the environmental impacts, *L. fortunei* is related to processes such as cyanobacterial blooms, nutrient recycling and increased transparency of the water column (Boltovskoy et al., 2009; Gazulha et al., 2012; Boltovskoy & Correa, 2015).

The observed impact is due to several characteristics that guarantee the success of golden mussels as invaders, such as: a short life cycle, rapid growth capacity and high fertility, as well as broad physiological tolerance to various abiotic factors such as temperature, salinity and oxygen. These characteristics allow *L. fortunei* to survive in humid areas, lakes, and other water courses (Morton, 1977; Campos et al., 2014; Oliveira t al., 2014). *Limnoperna fortunei* is gonochoric, with external fertilization, and its gametogenic cycle is controlled by environmental factors, especially temperature (Darrigran et al., 1999). Geographical dispersal of *L. fortunei* is largely facilitated by its planktonic larvae, which normally have a density between 6000 and 7000 ind/m^3 during peak reproductive months in South American waters (Cataldo & Boltovskoy, 2000; Boltovskoy et al., 2015).

The control and management of the invasive species depends on the knowledge of its reproductive aspects (Dei Tos et al., 2016) and the identification of *L. fortunei* larvae are essential for early detection of a new occurrence of invasion. However, the identification of bivalve larvae by optical methods and based on morphological characters becomes a

challenge and can generate erroneous reports about the invasion scenario (Garland & Zimmer, 2002; Hendriks et al., 2005). Thus, detection methods that corroborate optical methods have been used to diagnose the biological invasion of *L. fortunei*, especially molecular methods (de Paula et al., 2020). Polymerase Chain Reaction (PCR) and quantitative PCR (qPCR) assays have been used as the main molecular methods for identifying *L. fortunei* over the years (Pie et al., 2006; Boeger et al., 2007; Pie et al., 2017; Ribolli et al., 2021). Recently, approaches that use *L. fortunei eDNA* as well as nucleotide and protein sequences generated by ‘‘omics’’ technologies also allow the search for possible new molecular targets that help in the control of this invasive species (Uliano-Silva et al., 2014; Uliano-Silva et al., 2015; Uliano Silva et al., 2018; Ito et al., 2021; Andrade et al., 2021).

To date, all molecular techniques used to identify *L. fortunei* depend on *in vitro* tests. These laboratories are often far from the sampling points in the investigated reservoir, which implies complex logistics, due to the maintenance of these samples under specific conditions for the preservation of the genetic material, and, consequently, increasing the response time on the invasion, which is not so fast as needed (Williams et al; 2017). Furthermore, for detection of invasive species in very low abundance, sample concentration is often necessary. However, sample concentration can also lead to the simultaneous concentration of substrates inhibiting Taq polymerases used in PCR-based *eDNA* assays (Harvey et al., 2009). A viable alternative is the use of isothermal amplification, which has polymerases that are less susceptible to the action of PCR inhibitors (Koloren et al., 2011). Its main advantages over PCR include being performed at constant temperature, greater specificity and the possibility of detection with the naked eye through observation of turbidity or through the use of intercalating dyes to measure fluorescence in real-time assays (Zanoli & Spoto, 2012). These characteristics are relevant when considering the possibility of carrying out an *in situ* analysis (Carvalho et al., 2021). Isothermal amplification has been used to

detect invasive North American mussels *Dreissena polymorpha* (Pallas 1771) and *Dreissena bugensis* (Andrusov 1897) (Williams et al; 2017; Carvalho et al., 2021).

Thus, in this work, the loop-mediated isothermal amplification (LAMP) method was standardized in order to identify *L. fortunei*. Although the standardization presented here was performed under controlled laboratory conditions, these results may indicate a future application of the LAMP method to detect the *L. fortunei* directly in the field.

2 Material and Methods

2.1 Sampling

Adults of *Limnoperna fortunei* were collected manually at the fish farming station of the Volta Grande hydroelectric plant (HPP-Volta Grande), Minas Gerais, Brazil (20°01'49" S, 48°12'59" W). 20 water samples containing +30 bivalve larvae each were collected at the fish farming station of HPP-Volta Grande and transported to the laboratory, according to the protocol as described by Ribolli et al. (2021). The authorization for activities with scientific purpose is registered in the SISBIO (Sistema de Autorização e Informação em Biodiversidade) with the access code 72222-2.

2.2 DNA extraction

Tissues from three adults of *L. fortunei* were subjected to total DNA extraction using the E.Z.N.A® Mollusc DNA Kit (Omega Bio-tek, Norcross, GA), according to the manufacturer's instructions. For samples with larvae, total DNA extraction was performed using the E.Z.N.A® Water DNA Kit (Omega Bio-tek) kit manufacturer's instructions. DNA quantification and quality were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). All molecular assays related to the genetic heritage of the organisms used in this study are duly in accordance with the SisGen (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado)

standards and registered with the access code A118152.

2.3 Primer design

The upload of the raw sequencing data from the genome of *L. fortunei* deposited in GenBank (Access #ASM313041v1) was screened in order to detect the annotated transposition elements (the high content of these elements in the genome of *L. fortunei* is an efficient target for the DNA detection of this invader). Three sequences of repetitive elements were identified and BLASTs were performed against all NBCI genomes, so that the hits resemble other molluscs, but with less than 65% similarity. Based on their repeatability, these sequences are distributed in several *loci* in the *L. fortunei* genome. Only alignments with scores greater than 100 were selected, which confirms the repeatability of the sequence across multiple *loci*.

The primers were designed using the Primer Explorer V5 software and three sets were obtained, of which Set 1 (Table 1) showed the highest sensitivity and, therefore, was used to perform most of the tests described below. The primers sequences of Sets 2 and 3 are represented in Table S1.

2.4 Loop-mediated isothermal amplification (LAMP) for L. fortunei detection

The LAMP assay mixture contained 1 µL of 320 U/mL Bst Polymerase 2.0 WarmStart (New England Biolabs, Ipswich, MA), 2.5 µL of 10X Isothermal Amplification Buffer with 2 mM $MgSO_4$ (New England Biolabs), 1.5 µL of 6 mM $MgSO_4$ (8 mM total), 3.5 µL of dNTP Mix (1.4 mM each), 1 µL of FIP/BIP primers (1.6 µM each), 1 µL of F3/B3 primers (0.2 µM each), 1 µL of LoopF/LoopB (0.4 µM each), the amount of DNA was variable according to the specific assays (which will be described below), and final volume adjusted to 25 µL with nuclease-free water. Incubation for amplification was performed using an isothermal protocol at 65 ºC in a water bath, with time varying according to the specific experiments

(described below), followed by 20 min at 80 ºC for enzyme denaturation. For visualization, 1 µL of SYBR® Green I Nucleic Acid Stain 10,000X concentrated (Lonza Bioscience, Morrisville, NC) was added directly to the tube and placed under UV light. Confirmation of the amplification seen in the tubes was observed on 2% agarose gel.

To avoid contamination, it is worth mentioning that the DNA extraction, the preparation of the LAMP reaction mixture, and the incubation step for amplification took place in different rooms. Besides, the opening of the tubes for the addition of SYBR® Green occurred in a fume hood, and all assays involving adults of *L. fortunei* occurred in triplicates and with at least one day of difference between replicates and/or different assays.

2.4.1 Amplification sensitivity

Different amounts of total DNA: 10 ng, 1 ng, 0.1 ng, 0.01 ng, 0.001, and 0.0001 ng of adult *L. fortunei* were tested with each of the three primer Sets in LAMP assays for 60 min in a water bath, followed by the step of denaturation and visualization in UV light and agarose gel. From that point on, all assays listed below were performed with primer set 1.

2.4.2 Minimum time for reaction detection

Four samples containing 1 ng of adult *L. fortunei* DNA were kept in a water bath for 30, 60, 90 and 120 min, respectively. The samples were exposed to UV light and agarose gel to verify the amplification.

2.4.3 Cross-reactivity with other species

One ng of total DNA from adult *L. fortunei* and control DNA from molluscs and other organisms previously available in the laboratory were submitted to the LAMP assay for 60 min in a water bath, followed by denaturation and visualization under

UV light. The control species tested with Set 1 of primers were: Bivalvia - *Corbicula fluminea* (Müller 1774), *C. largillierti* (Philippi 1844), *D. polymorpha*, *D. bugensis*, Perna perna (Linnaeus 1758); Gastropoda - *Aplysia sp.*; and Cnidaria - *Cordylophora caspia* (Pallas 1771), a co-invasive species with *L. fortunei*.

2.4.4 LAMP from environmental samples

One ng of total DNA from each of 20 samples with larvae were subjected to the LAMP assay for 60 min, followed by denaturation and visualization under UV light. One ng of total DNA from adult *L. fortunei* was used as a positive control for the amplification.

2.5 qPCR

qPCR was conducted using specific primers Forward: 5'-GGGACTGGTTGGACAGTTTAT-3' and Reverse: 5'-ACGCACCAGCTAAATGAAGA-3' for the *L. fortunei* mitochondrial *COI* (*Cytochrome C Oxidase Subunit I*) (Pie et al. 2017). qPCR containing 1 ng of DNA from each one of the 20 environmental samples with larvae, 5 µL of iTAq™ Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), 1 µL of each primer at 10 µM, and final volume adjustment to 10 µL with nuclease-free water. Reactions occurred at 50 °C for 2 min, 95 °C for 2 min and 40 cycles of 94 °C for 15 s (denaturation step), and 65 °C for 1 min (annealing/extension) (Pie et L., 2017) in the Rotor GeneTM 3000 machine. The dissociation step to assess the specific melting temperature was carried out at the end of the amplification.

2.6 PCR

PCR was performed using specific primers Limno. COI F1: 5'-TTTAGAGTTAGCACGTCCTGGTAGGTT-3' and Limno. COI R1: 5'-

TCCAACCAGTCCCTACTCCACCCTCTA-3' for the *L. fortunei* mitochondrial *COI* gene which amplifies a ~300 bp (base pairs) fragment (Pie et al. 2006). PCR amplification was performed with 1 ng of DNA from each 20 environmental samples with larvae, 12.5 µL of PCR SuperMix (22 U/mL of recombinant Taq DNA polymerase, 22 mM Tris-HCl (pH 8,4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 µM of each dNTP, and stabilizers; Thermo Fisher Scientific, Carlsbad, CA), 1 µL of each primer at 10 µM, and the volume adjusted with nuclease-free water for final reaction of 20 µL. Cycling parameters comprised an initial denaturation of 5 min at 94 ºC, 30 rounds of amplification with 30 s at 94 ºC, 30 s at 58.2 ºC, 1 min at 72 ºC and a final extension of 72 ºC for 5 min (Pie et al., 2006). The results were visualized on 1% agarose gel.

3 Results

3.1 Validation of primers for tissue amplification

Concentrations from 10 ng to 0.0001 ng of DNA from adult tissues of *L. fortunei* were used with primer Set 1, and it was possible to obtain the sensitivity of the reaction from the direct amplification of bivalve tissues (Fig. 1). The colorimetric pattern revealed under UV light showed green staining (positive) (Fig. 1A), or high intensity of light (Fig. 1B), for reactions in which there was amplification (Fig. 1A/B 1-4) and orange staining (negative), or absence of light, indicating no amplification (Fig. 1A/B 5-6) or negative control (Fig. 1A/B NC). To corroborate the colorimetric result, the ladder-like amplification pattern can be observed on the agarose gel (Fig. 1C). This pattern was clearly observed in all samples that contained golden mussel DNA (Fig. 1C lanes 1-4). As the DNA concentration decreased in each reaction, the change from green to orange in the tubes was also observed, indicating that the lower amount of DNA available was correlated with the lower intensity of staining. The detection limit of primer Sets 2 and 3 was only sensitive up to 0.1 ng of DNA (Fig. S1).

3.2 Minimum time for amplification detection

To determine how long the tested primer set 1 could detect amplification positivity, four samples were left in reaction conditions between 30 and 120 min (Fig. 2). Amplification was detected after 60 minutes of reaction time, as shown in the green staining of tubes and samples from that time on (Fig. 2A/B 2-4). At 30 min of reaction there was no detection of amplification evidenced by the orange stain (Fig 2A/B 1), as observed in the respective negative controls of each reaction (Fig. 2A/B 1'-4'). According to the colorimetric results, the ladder-like amplification pattern was present only in the 60, 90 and 120 min samples (Fig. 2C lanes 2-4).

3.3 The primer set is species-specific for L. fortunei

To test whether the primer set used was specific for *L. fortunei*, cross-amplification reactions were performed using DNA from other species, including bivalves, other molluscs and invasive species that co-occur with the golden mussel (Fig. 3). The green color was exclusively observed in the reaction that contained the DNA of *L. fortunei* (Fig. 3A/B Lf). The reactions for other bivalve species, such as *C. fluminea* (Fig. 3A/B Cf), *C. largillierti* (Fig. 3A/B Cl), *D. polymorpha* (Fig. 3A/B Dp), *D. bugensis* (Fig 3A/B Db) and *P. perna* (Fig. 3 A/B Pp); for the gastropod *Aplysia sp.* (Fig. 3A/B A sp.); and for the cnidarian *C. caspia* (Fig. 3 A/B Cc), as well as for the negative control (Fig. 3A/B NC), they were all orange. Ladder-like amplification was also observed exclusively in the reaction in which *L. fortunei* DNA was present (Fig. 3C - lane Lf).

3.4 Validation of primers for amplification of environmental samples

To verify the amplification in environmental samples, 20 water samples containing bivalve larvae were subjected to the LAMP amplification reaction (Fig. 4). The green color was detected in all samples (Fig. 4A/B 1-20), and the orange color was observed only in the

negative reaction control (Fig. 4A/B NC). The total DNA of an adult *L. fortunei* was used as a positive control of the reaction, which also showed a green color (Fig. 4A/B +). Ladder-like amplification occurred for the 20 samples and the positive control (Fig. 4C 1-20/+).

To prove its viability, the 20 water samples were submitted to the qPCR and PCR protocols, the two current methods for laboratory detection of *L. fortunei* DNA from environmental samples (Fig. 4B/C). The detection of the *COI* gene by qPCR was confirmed for the 20 samples with larvae and the positive control, different from the absence of amplification of the negative control (Fig. 4B). The specificity of the primers used in qPCR was confirmed by the melting curve (Fig. 4B). *COI* gene amplification of ~300 bp fragment was also detected in all environmental samples and in the positive control (Fig. 4C lanes 1-20/+).

4 Discussion

Given the high dissemination capacity of *L. fortunei*, early detection becomes a priority for mitigation plans and control of the invasion process, favoring the development of biotechnological methods which could viabilize the detection of invaders in low numbers (Carvalho et al., 2021). In the case of invasive aquatic species, eDNA analysis appears to be one of the most effective methods to detect species that are in low numbers in a context of recent invasion (Ficetola et al., 2008; Jerde et al., 2011). Golden mussel detection methods using PCR have been implemented and improved over the years. However, to date and to the best of our knowledge, no LAMP method has been used to detect *L. fortunei* and we describe it for the first time here.

The standardization of a LAMP method is not intended to replace PCR and other laboratory tests for the identification of *L. fortunei*. There is a need to develop an integrated system to quickly detect the presence of invasive species and allow a quick response to contain and control this process, mainly by entities interested in the management of rivers, basins and other invaded regions (Silva et al., 2016). The LAMP method indicates that there

is a possibility of this quick detection of *L. fortunei*, even in low concentration, as observed from 60 minutes of DNA amplification (Fig. 2).

The detection limit of 0.01 ng of DNA adult from *L. fortunei* tissues was similar to the results obtained for the amplification of the *COI* of *D. bugensis*, which presented the same detection limit value, when a specific primer set for the *COI* gene of this invader was used (Williams et al., 2017). Interestingly, in this study from Williams et al. (2017) the detection limit for *D. polymorpha* was only 10 ng of DNA using a set of *COI* specific primers. However, when using a set of primers for 18S rRNA from *Dereissena sp.*, the detection limit presented was 0.1 ng per reaction, decreasing the value obtained for *COI* from *D. bugensis*, but increasing when compared to *COI* from *D. polymorpha*. These results indicate that the choice of the DNA target region and the designed primer set must be carefully determined and interpreted, adapting to what is expected when using the LAMP method. As new sets of primers are tested to detect *L. fortunei*, it is desirable that even lower concentrations of DNA be detected.

Even though we have a positive result for all collected field samples, confirmed by PCR and qPCR (Fig. 4), further studies should be conducted to verify the limited number of *L. fortunei* larvae that our primer set can detect. The ability of *D. polymorpha COI* primers to detect veliger larvae through LAMP from samples of the field was measured and revealed the detection of 0.09 veligers per reaction (Williams et al., 2017). However, our objective with the field samples was to evaluate the detection efficiency of our primers in environmental samples, since DNA can be susceptible to dilution effects, have low concentration or even be degraded due to intrinsic characteristics of water (temperature, salinity, pH, etc.) (Herder et al., 2014). Furthermore, in environments where there is co-occurrence between *L. fortunei* and *C. fluminea*, such as in the HPP-Volta Grande (da Silva Camargo et al., 2022), the LAMP method would be a viable technique for distinguishing the larvae of these bivalves, since our primers did not present a positive amplification for *C.*

fluminea (Fig. 3), and consequently, it would be a method capable of monitoring and reporting new invasions of *L. fortunei.*

Reporting invasions effectively and reliably becomes urgent, especially when considering the prevention and protection of areas not yet invaded. Barbosa et al. (2018) built a model that calculated the risk of *L. fortunei* invading the northern region of Brazil, between the years 2030 and 2050, however the forecasts of this model could be anticipated, since the presence of *L. fortunei* in HPP-Sobradinho was already documented. (Barbosa et al., 2016). This reinforces the need for rapid and early detection of *L. fortunei* combined with an efficient surveillance system. A possible invasion of *L. fortunei* to the Amazon basin is pointed out with concern and it is only a matter of time before it materializes (Ludwig et al., 2021; Petsch et al., 2021).

The aspects involving the biological invasion around *L. fortunei* are complex and demand the integration of multiple environmental, economic and social spheres in Brazil. It is important to involve multiple actors and methodological approaches to prevent, combat and mitigate the spread of *L. fortunei* (de Paula et al., 2021). Obviously, new studies should be conducted in order to implement, improve and evaluate the effectiveness of LAMP for the detection of *L. fortunei* in other sceneries, especially in the case of samples from other invaded (or non-invaded) environments, or with low number and concentration of DNA. In addition, studies should be developed to enable the application of the field-based *in situ* LAMP method for the *L. fortunei* detection.

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animals and water samples with the larvae used in this work.

6 Disclosure statement

No potential conflict of interest was reported by the author(s).

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9 Figures

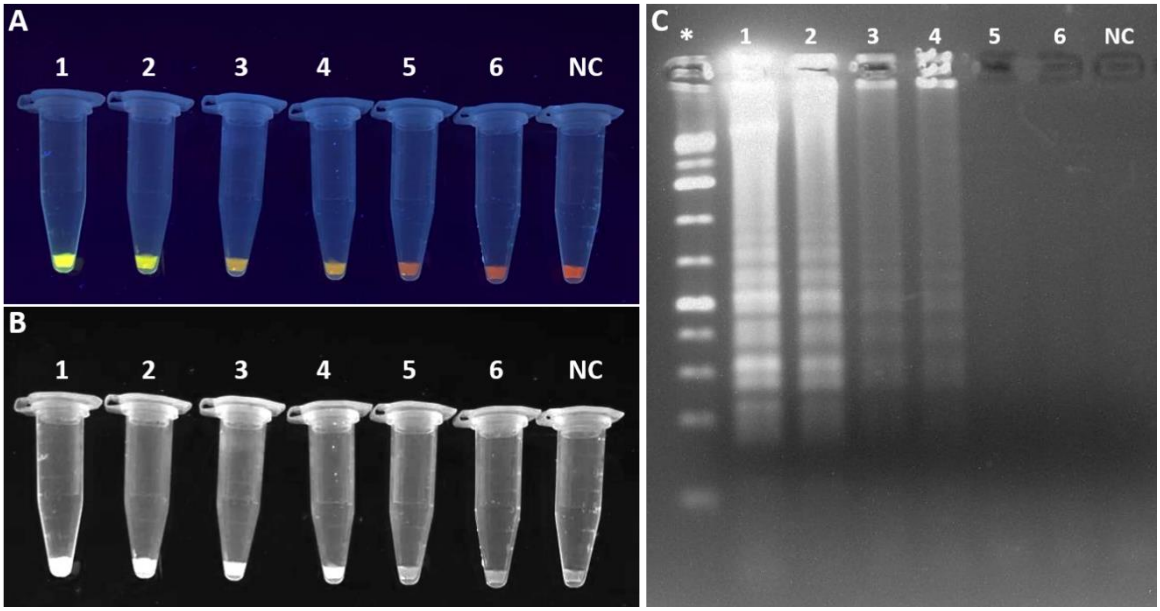

Figure 1. Limit of detection of *Limnoperna fortunei* by LAMP. (A) Green staining (positive), or **(B)** high intensity of light, for reactions in which there was amplification and orange staining (negative), or absence of light intensity, indicating absence of amplification and negative control. **(C)** The ladder-like amplification pattern was observed for samples that had positive amplification. Samples 1, 2, 3, 4, 5, 6 contained 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng of DNA, respectively; **NC:** non-template control; ***:** 1 Kb Plus DNA Ladder (Thermo Fisher Scientific).

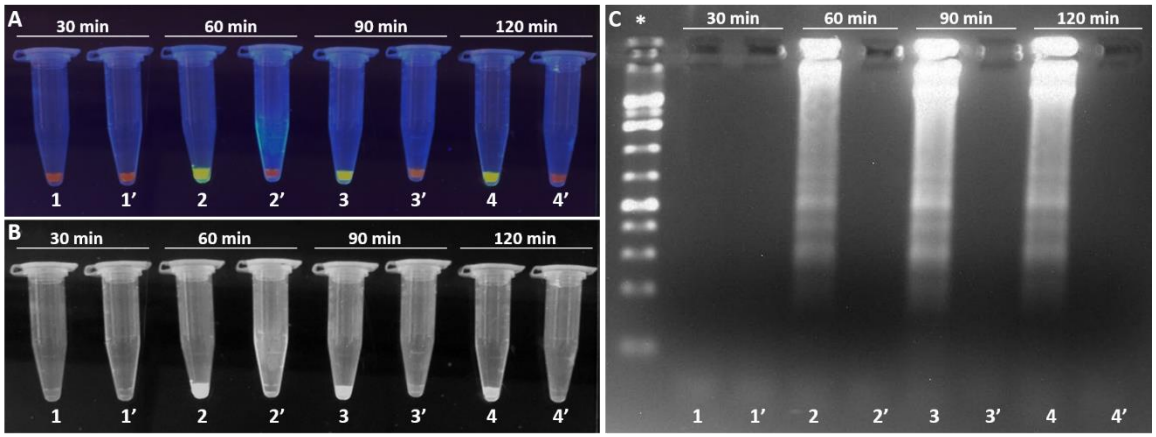

Figure 2. Minimum time for detection of *Limnoperna fortunei* by LAMP. (A) Amplification was detected after 60 minutes of reaction time, as shown in the green staining, **(B)** high intensity of light of tubes and samples from that time on. At 30 min of reaction there was no detection of amplification evidenced by the orange stain, or absence of light intensity. **(C)** The ladder-like amplification pattern was observed for samples that had positive amplification. All samples 1, 2, 3, 4, 5, 6 contained 1 ng of DNA, and 1', 2', 3, '4' were the respective negative controls at each time tested; **NC:** non-template control; ***:** 1 Kb Plus DNA Ladder (Thermo Fisher Scientific).

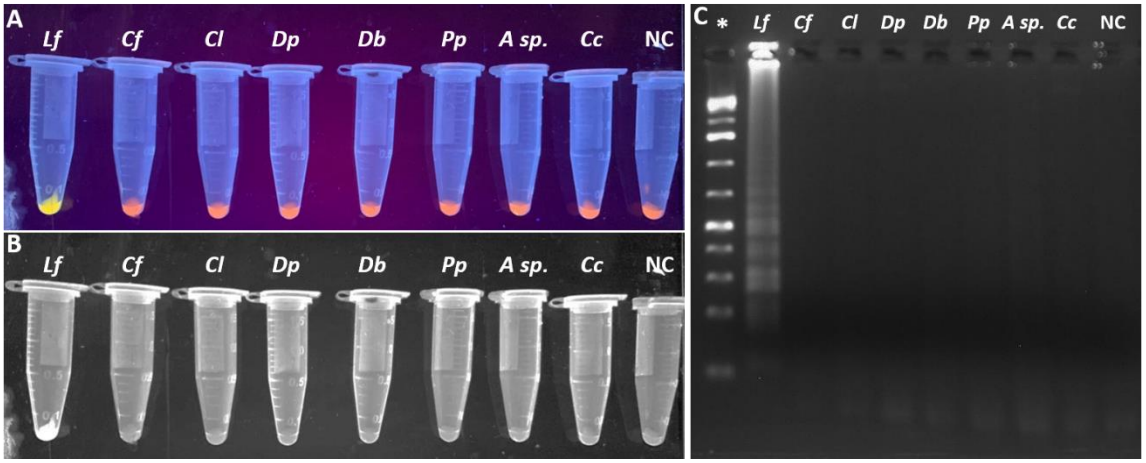

Figure 3. The primer set is species-specific for *Limnoperna fortunei* detection by LAMP. (A) The green color, **(B)** and high intensity of light, was only observed exclusively in the reaction that contained the DNA of *L. fortunei*. The other reactions for other species were all orange, showing a lack of amplification. **(C)** The ladder-like amplification pattern was observed only for samples with DNA from *L. fortunei*. **Lf:** *Limnoperna fortunei*, **Cf:** *Corbicula fluminea*, **Cl:** *Corbicula largillierti*, **Dp:** *Dreissena polymorpha*, **Db:** *Dreissena bugensis*, **Pp:** *Perna perna*, **A sp.**: *Aplysia sp.*, **Cc:** *Cordylophora caspia*; **NC:** non-template control; ***:** 1 Kb Plus DNA Ladder (Thermo Fisher Scientific).

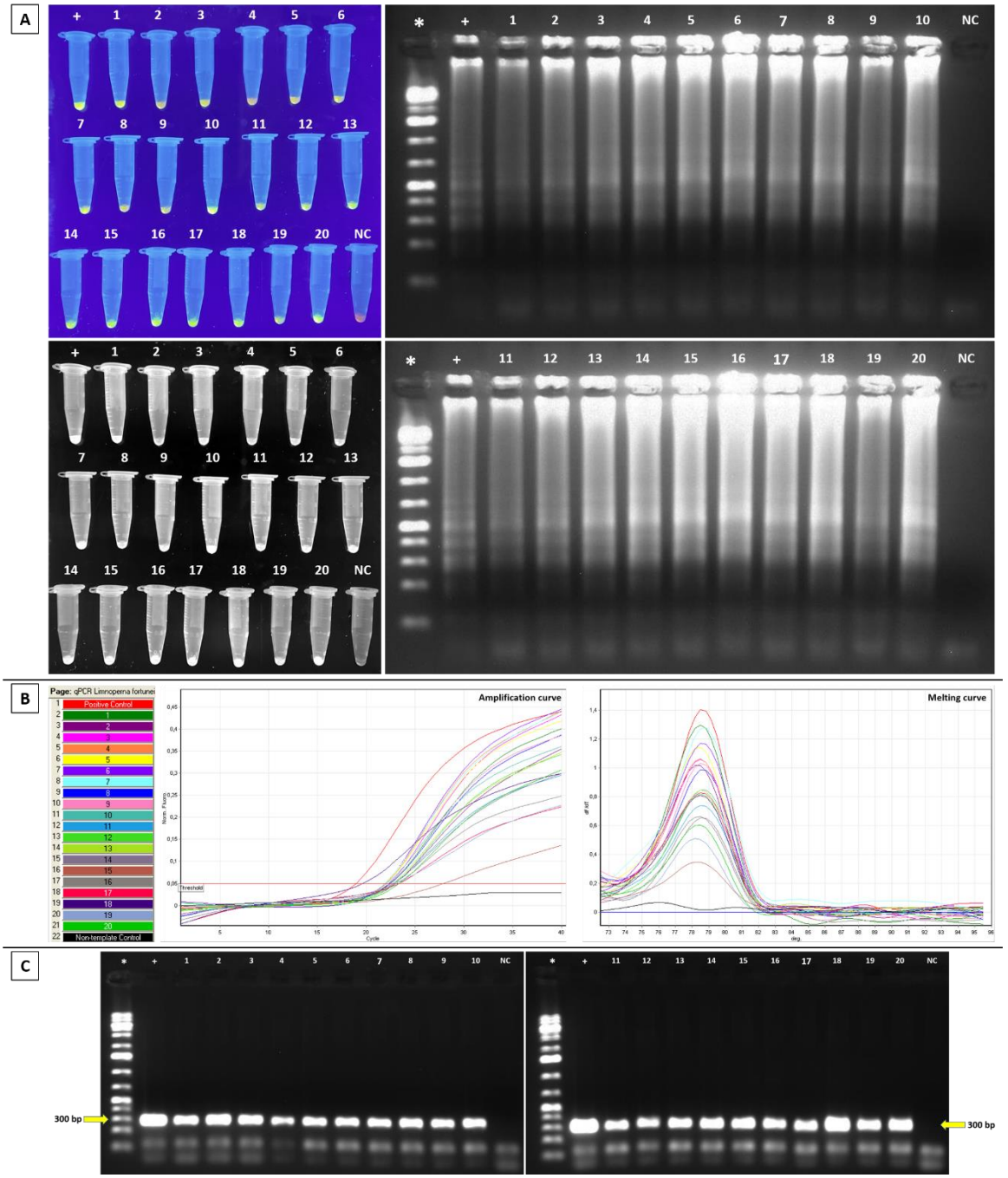

Figure 4. Amplification of Limnoperna fortunei by LAMP from environmental samples. **(A)** The green color, or high-intensity light, was detected in each of the tubes corresponding to the 20 samples collected and positive control, and the orange color was observed only in the negative control. Ladder-like amplification occurred for both the 20 samples and the positive control. **(B)** Amplification of the 20 environmental samples by qPCR**. (C)** Amplification of the 20 environmental samples by PCR with an expected amplicon with ~300 pb. Samples 1 to 20 each contained 1 ng of total DNA; **+:** positive control; **NC:** non-template control; ***:** 1 Kb Plus DNA Ladder (Thermo Fisher Scientific); **bp:** base pairs.

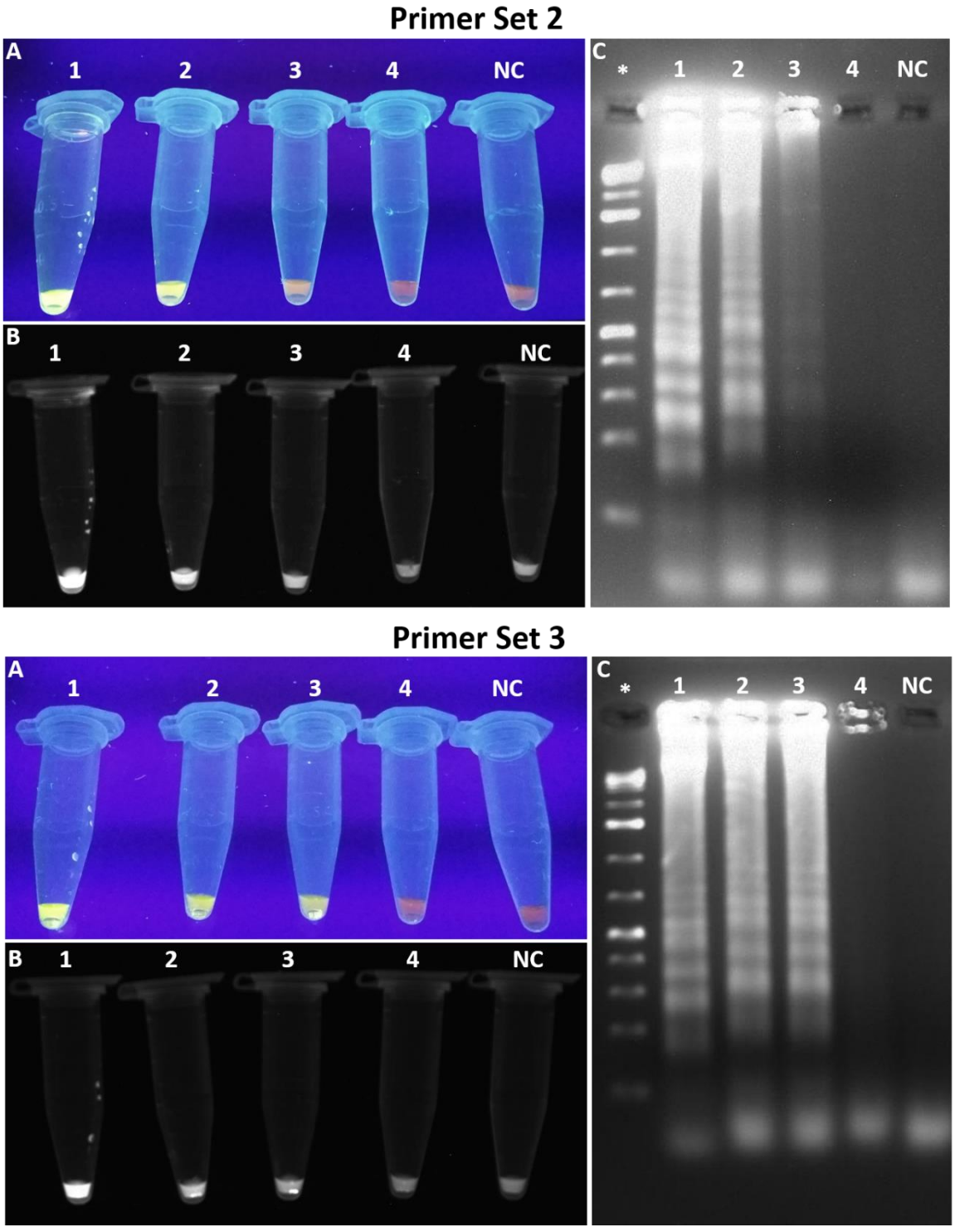

Figure S1. Limit of detection of *Limnoperna fortunei* by LAMP with alternative sets of primers. Set 2 and 3: (A) Green staining (positive), or **(B)** high intensity of light, for reactions in which there was amplification and orange staining (negative), or absence of light intensity, indicating absence of amplification and for negative control. **(C)** The ladder-like amplification pattern was observed for samples that had positive amplification. Samples 1, 2, 3, 4 contained 10, 1, 0.1, and 0.01 ng of DNA, respectively; **NC:** non-template control; ***:** 1 Kb Plus DNA Ladder (Thermo Fisher Scientific).

Tables

Table 1. LAMP primer set used for detection of *Limnoperna fortunei*. F3/B3: outer primers; FIP/BIP: inner primers; LF/BF: loop-primers.

Primer	Sequence
Set1_Lfor_F3	ACATGTTTTTCAACAGACTGT
Set1_Lfor_B3	GGTAAATTGAATGTAAATAGTCACC
Set1_Lfor_FIP	AACTCAGCCTCATAAGAGTACAAAATATTCCAATGGGTACTAATTGTG
Set1_Lfor_BIP	CCTAGAAGGCCTACTGAAGAAGAAGACAAAACATCATCAATATAGCGG
Set1_Lfor_LF	ACAGGTCGGCGAGGAGA
Set1_Lfor_LB	AGACTCTGGCCCAGACCTTTA

Table S1. Alternative sets of LAMP primers for detection of *Limnoperna fortunei*. F3/B3: outer primers; FIP/BIP: inner primers; LF/BF: loop-primers.

Set primer 2	
Primer	**Sequence**
Set2_Lfor_F3	CATTAGACTGTGGCCTGTA
Set2_Lfor_B3	TCACCAAGTGGACTGAGG
Set2_Lfor_FIP	CAAAGGGACAAGCTTTGAGTCAGACTTGTAGTCCTAGTTTTGGCT
Set2_Lfor_BIP	GCTGGAGGGGTGTTCCAAATACAAGAAAGCATAATTGTAGCA
Set2_Lfor_LF	GACATGTGTAATCTCTTGGGC
Set2_Lfor_LB	AGTTGTCAATGAGGGCAGT
Set primer 3	
Primer	**Sequence**
Set3_Lfor_F3	GAAGGCTCGTTTGCTTCT
Set3_Lfor_B3	TTTTAAAAGTCGTAGCCAGC
Set3_Lfor_FIP	GCAAGTCATACGTCAAAATCCTTGACAGGTGTGGTTCTAAATGCT
Set3_Lfor_BIP	GCAGAACCATAAATAAAACAACCGTTGAATGCGATGTTTTACTCAA
Set3_Lfor_LF	CAAGGATTGCGACTATGCC
Set3_Lfor_LB	AATCCAATTTCGATCGAACCAG

8. Conclusões Gerais

O desenvolvimento deste trabalho de tese permitiu concluir que (de acordo com os capítulos apresentados):

Capítulo 1

- Os gêneros *Gallionella* e *Ferrigenium*, pertencentes à Gallionellaceae, foram os mais ubíquos na UHE-Irapé;
- A caverna de percolagem apresentou-se como um *hotspot* para as FeOB neutrofílicas por abrigar maior diversidade de gêneros da família Gallionellaceae, incluindo *Gallionella*, *Ferriphaselus*, *Sideroxydans* e *Candidatus* Nitrotoga;
- As bioassinaturas foram encontradas apenas na caverna de percolagem, de modo que os *stalks* foram produzidos, provavelmente, por *Ferriphaselus* e as *sheaths* produzidas, possivelmente, por *Crenothrix*.

Capítulo 2

- Foi possível atualizar a distribuição de *C. caspia* no Brasil e registrar a primeira ocorrência desse invasor nos estados de Goiás e Minas Gerais;
- Foram geradas as primeiras sequências genéticas brasileiras de *C. caspia*;
- Não há um padrão claro de especialização ecofisiológica dos clados principais de *C. caspia* em relação à salinidade;
- Altos níveis de diferenciação genética entre amostras brasileiras suportam a existência de múltiplas espécies crípticas dentro do gênero *Cordylophora*;
- A análise de reconstrução do estado ancestral sugeriu uma origem presumida de água doce para *C. caspia*.

Capítulo 3

- A compilação de trabalhos sobre os aspectos moleculares acerca do processo de invasão ocasionado por *L. fortunei* permitiu direcionar e propor projetos que detectassem precocemente um novo quadro de invasão;
- O panorama envolvendo a invasão biológica de *L. fortunei* dependem da integração das esferas pública e privada, no tocante às questões ambiental, econômica e social, com a participação de diferentes atores;

- Foi padronizada, ineditamente, a LAMP-PCR para a detecção de *L. fortunei* em condições laboratoriais.
- A LAMP mostrou-se sensível a baixas concentrações de DNA de *L. fortunei*;
- Determinou-se a estimativa de tempo mínima para que a LAMP detecte DNA de *L. fortunei*;
- O set de *primers* desenhados para LAMP amplificaram apenas DNA de *L. fortunei* e não tiveram reações cruzadas com outras espécies;
- A técnica de LAMP amplificou o DNA extraído de amostras ambientais coletadas em campo.

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APÊNDICES

I. Atividades de Pesquisa

Desde a seleção para ingresso no doutorado (fevereiro/2018), o aluno colaborou com a produção de quatro artigos científicos, sendo alguns deles com a coparticipação da orientadora e da coorientadora, envolvendo estudos relativos a questões acerca das espécies bioincrustantes, além de colaborações com outros grupos do Programa de Pós-graduação em Biologia Celular (PPG-BioCel). São eles (em ordem cronológica de publicação):

I.A. Oliveira Junior, Renato Brito De; **Paula, Rayan Silva De**; Diniz, Vinícius Sergio Rodrigues; Carvalho, Marcela David De; Cardoso, Antônio Valadão. *RFLP pattern determination for the invasive bivalves Limnoperna fortunei (Dunker, 1857) and Corbicula fluminea (Muller, 1774)*. Revista Ambiente e Água, v. 13, p. 1-9, 2018. https://doi.org/10.4136/ambi-agua.2172

I.B. Valadão, Priscila Aparecida Costa; De Aragão, Bárbara Campos; Andrade, Jéssica Neves; Magalhães-Gomes, Matheus Proença S.; Foureaux, Giselle; Joviano-Santos, Julliane Vasconcelos; Nogueira, José Carlos; Machado, Thatiane Cristina Gonçalves; De Jesus, Itamar Couto Guedes; Nogueira, Julia Meireles; **De Paula, Rayan Silva**; Peixoto, Luisa; Ribeiro, Fabíola Mara; Tapia, Juan Carlos; **Jorge, Erika Cristina**; Guatimosim, Silvia; Guatimosim, Cristina. *Abnormalities in the Motor Unit of a Fast-Twitch Lower Limb Skeletal Muscle in Huntington's Disease*. ASN Neuro, v. 11, p. 1-20, 2019. https://doi.org/10.1177/1759091419886212

I.C. **Reis, Mariana De Paula**; **De Paula, Rayan Silva**; Reis, André Luiz Martins; Souza, Clara Carvalho E; Júnior, Renato Brito De Oliveira; Ferreira, Jacqueline Alves; Mota, Helen Regina; De Carvalho, Marcela David; **Jorge, Erika Cristina**; Cardoso, Antônio Valadão; Nascimento, Andréa Maria Amaral. *Microbial composition of a hydropower cooling water system reveals thermophilic bacteria with a possible role in primary biofilm formation*. Biofouling, v. 37, p. 246-256, 2021. https://doi.org/10.1080/08927014.2021.1897790

I.D. Freitas, Erico Tadeu Fraga; Moreira, Amanda Maria Siqueira; **De Paula, Rayan Silva**; Andrade, Gabriela Rabelo; De Carvalho, Marcela David; Assis, Paulo Santos; **Jorge, Erika Cristina**; Cardoso, Antônio Valadão. *Ultrastructure of the gill ciliary epithelium of Limnoperna fortunei (Dunker 1857), the invasive golden mussel*. Bmc Zoology, v. 7, p. 6, 2022. https://doi.org/10.1186/s40850-022-00107-y

II. Atividades de Extensão

O aluno integrou o projeto de extensão “Modelos tridimensionais de Embriologia: Uma produção didática da UFMG como instrumento de transformação e impacto na formação docente e discente por todo país” (2018-2021; SIEX 400522) coordenado pela Profa. Dra. Gerluza Aparecida Borges Silva (PPG-BioCel), cujo objetivo é disponibilizar para a(s) escola(s) participante(s) um método imersivo de ensinar Embriologia atrelada a importantes aspectos da sexualidade pré-adolescente. Este trabalho foi reconhecido e premiado em três eventos distintos:

II.A. Menção Honrosa, I Jornada Científica de Ensino Extensão, Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais. 2018.

II.B. Melhor Trabalho na Categoria Extensão, 4º Encontro de Ciência, Ensino e Cultura do ICB/UFMG, Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais. 2018.

II.C. Menção Honrosa de Extensão, XXII Encontro da Extensão/PROEX , Semana do Conhecimento, Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais. 2019.

O aluno participou do Projeto de Extensão “Curious Minds” (2017-2018; SIEX 402913) coordenado pela Profa Dra. Walderez Ornelas Dutra (PPG-BioCel), cujo objetivo foi inserir alunos do ensino médio à vivência laboratorial por meio do desenvolvimento do pensamento científico e de realização de práticas experimentais de forma ativa. Esse projeto culminou em publicação de um artigo científico:

II.D. PAULA, Rayan Silva De; NOGUEIRA, Júlia Meireles; COSTA, Alinne Do Carmo; COPOLA, Aline Gonçalves Lio; **JORGE, Erika Cristina**; DUTRA, Walderez Ornelas. *Curious Minds: Aproximação do Contexto Escolar à Realidade Laboratorial das Universidades.* Revista MultiAtual, v. 1, p. 39-54, 2020. https://doi.org/10.5281/zenodo.465517

O aluno também participou do Projeto de Extensão *Workshops* para Desenvolvimento de Habilidades para Jovens Cientistas (2020-2021; SIEX: 203627) coordenado pelo Prof. Diogo Montes Vidal, cujo principal objetivo foi promover dois eventos de divulgação científica em um projeto de extensão junto à equipe do *ASC Student Chapter* UFMG do Departamento de Química do Instituto de Ciências Exatas da UFMG.

III. Atividades de Ensino

O aluno cumpriu cursou as quatro disciplinas de Estágios Didáticos coordenados pela Profa. Dra. Cleida Aparecida (PPG-BioCel) que são disponibilizados pelo programa, recebendo um certificado de Professor-Estagiário em Embriologia em que nas disciplinas de Embriologia ofertadas pelo Departamento de Morfologia do Instituto de Ciências Biológicas para as turmas de Ciências Biológicas (2º/2017-1º/2019) e Biomedicina (1º/2019).

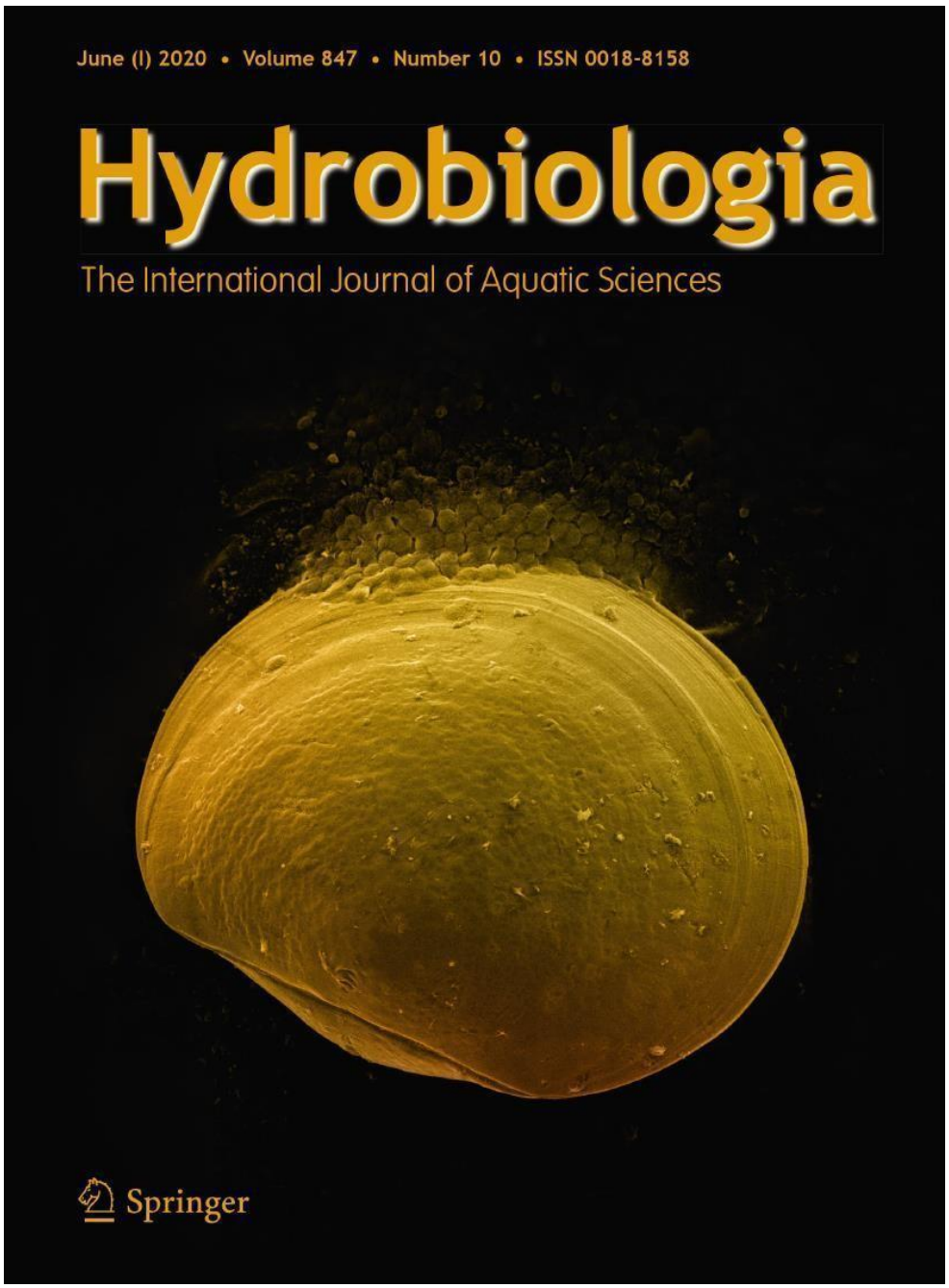
Desde o segundo semestre de 2019 foi Professor Voluntário no Setor de Embriologia do Departamento de Morfologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais. Ministrou aulas de Embriologia Geral para os cursos de Enfermagem (30h/2º_2019) e Biomedicina (30h/1º_2020), Embriologia Aplicada à Fonoaudiologia (45h/1º-2º_2020) para o curso de Fonoaudiologia, e Embriologia Aplicada à Medicina Veterinária (30h/ 2º_2020) para o curso de Medicina Veterinária.

Ministrou o curso Tópicos Especiais em Biologia Molecular (2020) para a Pós-graduação em Medicina Veterinária da a Universidade de Uberaba (Uniube), junto dos Professores Bruno Machado Bertassoli e Júlia Meireles Nogueira.

Atuou como Professor Convidado do Centro Universitário de Belo Horizonte (UniBH) para ministrar aulas da disciplina Sistemas Vitais dos Animais para o curso de Medicina Veterinária, a convite do Professor Bruno Machado Bertassoli.

IV. Capa da Revista Hydrobiologia

O artigo de revisão "*Genetic and functional repertoires of Limnoperna fortunei (Dunker, 1857) (Mollusca, Mytilidae): a review on the use of molecular techniques for the detection and control of the golden mussel*", publicado na revista Hydrobioligia (e apresentado como um dos capítulos dessa Tese de Doutorado), foi selecionado para ser capa do Volume 847, Número10, em junho de 2020.



Cover illustration: *Straight-hinged veliger of the golden mussel (Limnoperna fortunei) on a carbon substrate under Scanning Electron Microscopy (SEM), digitally coloured. SEM Magnification: 3.10kx, HV: 10kV, WD: 17.02mm. Equipment: Vega 3 Tescan. Image courtesy of Arthur Corrêa de Almeida*[*]*, Anna Carolina Paganini Guañabens*[*] *& Gabriela Rabelo Andrade*[*] *(*[*]*2014, Bioengineering Centre of Invasive Species (CBEIH), Brazil – www.cbeih.org) (Hydrobiologia 847(10): 2193–2202).*

V. Licença do CEBIH para realização de coleta de espécies invasoras aquáticas

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 72222-2	Data da Emissão: 17/06/2020 08:58:05	Data da Revalidação*: 24/09/2020
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Paulo Santos Assis	CPF: 130.740.246-15
Título do Projeto: Controle do Mexilha?o Dourado: Bioengenharia e Novos Materiais para Aplicac?o?es em Ecossistemas e Usinas Hidrele?tricas-Fase 2	
Nome da Instituição: UNIVERSIDADE FEDERAL DE OURO PRETO	CNPJ: 23.070.659/0001-10

Locais onde as atividades de campo serão executadas

#	Descrição do local	Município-UF	Bioma	Caverna?	Tipo
1	-19.015468, -50.493776	Santa Vitória-MG	Mata Atlântica	Não	Fora de UC Federal
2	-20.032239, -48.221533	Conceição das Alagoas-MG	Cerrado	Não	Dentro de UC Estadual

Atividades

#	Atividade	Grupo de Atividade
1	Coleta/transporte de espécimes da fauna silvestre in situ	Fora de UC Federal
2	Coleta/transporte de amostras biológicas in situ	Fora de UC Federal
3	Manutenção temporária (até 24 meses) de invertebrados silvestres em cativeiro	Atividades ex-situ (fora da natureza)

Atividades X Táxons

#	Atividade	Táxon	Qtde.
1	Manutenção temporária (até 24 meses) de invertebrados silvestres em cativeiro	Cordylophora caspia	-
2	Coleta/transporte de amostras biológicas in situ	Cordylophora caspia	-
3	Coleta/transporte de espécimes da fauna silvestre in situ	Cordylophora caspia	5000
4	Coleta/transporte de espécimes da fauna silvestre in situ	Limnoperna fortunei	5000
5	Manutenção temporária (até 24 meses) de invertebrados silvestres em cativeiro	Limnoperna fortunei	-
6	Coleta/transporte de amostras biológicas in situ	Limnoperna fortunei	-

Materiais e Métodos

#	Tipo de Método (Grupo taxonômico)	Materiais
1	Amostras biológicas (Invertebrados Aquáticos)	Outras amostras biológicas(Larvas planctônicas)
2	Método de captura/coleta (Invertebrados Aquáticos)	Coleta manual, Peneira, Puçá, Rede de arrasto de fundo (tração motorizada): tangones, portas ou parelha, Rede de plâncton

Este documento foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

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