BIANCA LAÍS ZIMMERMANN

EVOLUÇÃO EM SIMBIOSE: ESTUDO DE CASO DA RELAÇÃO ENTRE AS BACTÉRIAS
***Wolbachia* (Alphaproteobacteria, Rickettsiales) E OS ISÓPODOS TERRESTRES**
(Crustacea, Oniscidea) NA AMÉRICA DO SUL

Tese apresentada ao Programa de Pós-Graduação em Biologia Animal, Instituto de Biociências, da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do título de Doutora em Biologia Animal.

Área de Concentração: Biodiversidade

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UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PORTO ALEGRE

2014

EVOLUÇÃO EM SIMBIOSE: ESTUDO DE CASO DA RELAÇÃO ENTRE AS BACTÉRIAS *Wolbachia* (Alphaproteobacteria, Rickettsiales) E OS ISÓPODOS TERRESTRES (Crustacea, Oniscidea) NA AMÉRICA DO SUL

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"No mistério do sem-fim

equilibra-se um planeta.

E, no planeta, um jardim,

e, no jardim, um canteiro;

no canteiro uma violeta,

e, sobre ela, o dia inteiro,

entre o planeta e o sem-fim,

a asa de uma borboleta."

Cecília Meireles

Agradecimentos

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Prefácio

Desde o advento das técnicas moleculares, no final da década de 1980, os estudos com as bactérias *Wolbachia* têm aumentado de forma exponencial. E não era para menos, pois uma bactéria até então considerada de menor importância, tem se mostrado como o mais abundante e cosmopolita endossimbionte do planeta Terra, o qual influencia de forma drástica e determinante a biologia e, consequentemente, a evolução de seus hospedeiros artrópodos e nematoides. Atualmente, os estudos sobre *Wolbachia* assumiram tamanha importância, que podem ser a chave para o controle de doenças responsáveis pela morte de milhões de pessoas em todo mundo.

A relação simbiótica entre *Wolbachia* e seus hospedeiros isópodos terrestres é conhecida há bastante tempo e relativamente bem estudada na Europa. No entanto, na América do Sul e região Neotropical, os estudos são ínfimos e muito pouco se sabe sobre o tema. A presente tese tenta suprir, de forma singela, essa brecha no conhecimento, apresentando os esforços no sentido de identificar as espécies infectadas, compreender a diversidade e relações filogenéticas das linhagens encontradas, além de verificar os efeitos da bactéria na aptidão de seus hospedeiros. Pode-se dizer que os resultados obtidos superaram nossas expectativas, pois um cenário evolutivo totalmente novo foi observado na região Neotropical, demonstrando quão complexa e fascinante é a relação entre *Wolbachia* e os isópodos terrestres.

A tese é composta por quatro capítulos apresentados na forma de artigos científicos. Esses foram redigidos de acordo com as normas dos periódicos aos quais foram (ou serão) submetidos. Inicialmente, é apresentada a seção "Introdução Geral", que serve para situar o leitor sobre o atual nível de conhecimento dos temas abordados nesta tese. O capítulo I, artigo publicado no ano de 2012, apresenta os primeiros registros de infecção por *Wolbachia* em espécies de isópodos terrestres na América do Sul. No capítulo II são investigados os efeitos da bactéria sobre a aptidão dos hospedeiros naturalmente infectados da espécie *Atlantoscia petronioi*. O capítulo III, por sua vez, diz respeito ao maior *screening* já realizado na região Neotropical para detecção da presença e diversidade de *Wolbachia* em populações naturais de isópodos terrestres. O mesmo traz inúmeras novidades e resultados nunca antes observados para o grupo em questão. O quarto e último capítulo, cujos motivos da elaboração são justificados no fim da introdução geral, trata da filogenia molecular do gênero *Atlantoscia*. Finalmente, comentários gerais, conclusões e perspectivas são abordados no tópico "Considerações Finais".

Resumo

As bactérias *Wolbachia* são conhecidas por infectar uma variedade de artrópodos e nematoides. Em artrópodos, a presença da bactéria já foi detectada em uma quantidade impressionante de insetos, crustáceos e quelicerados, o que faz de *Wolbachia* o mais abundante endossimbionte do planeta Terra. A relação simbiótica entre *Wolbachia* e seus hospedeiros isópodos terrestres é conhecida há bastante tempo e relativamente bem estudada na Europa. No entanto, na América do Sul e região Neotropical, os estudos são ínfimos e muito pouco se sabe sobre o tema. Os objetivos da presente tese foram: examinar a ocorrência e prevalência de infecção de *Wolbachia* em populações naturais de isópodos terrestres da América do Sul; analisar a diversidade genética e as relações filogenéticas das linhagens encontradas; verificar a influência da bactéria sobre a aptidão de *Atlantoscia petronioi*; além de realizar a filogenia molecular do gênero Neotropical *Atlantoscia*. Depois do primeiro registro da infecção em três espécies (duas nativas e uma introduzida) de isópodos terrestres da América do Sul, um *screening* mais amplo foi realizado. Animais foram coletados em 11 estados brasileiros, além de municípios da Argentina e Uruguai. A identificação molecular da presença da infecção foi realizada com o gene *16S rDNA*, e as amostras positivas também foram testadas com os genes *dnaA* e aqueles da MLST (*Multilocus Sequencing Typing - coxA*, *gatB*, *hcpA*, *fbpA*, *ftsZ*). Um total de 1172 indivíduos, representando 11 famílias e 35 espécies (26 nativas e nove introduzidas) foi testado para a presença de *Wolbachia*, sendo que 16 espécies foram positivas e em oito destas, este é o primeiro registro de infecção. Apesar da baixa prevalência geral de infecção, as linhagens encontradas nas espécies nativas apresentaram alta diversidade e, apesar da maioria fazer parte do supergrupo B, nenhuma delas faz parte do *Oni*clado. Pela primeira vez, linhagens dos supergrupos A e F foram registradas para isópodos terrestres, evidenciando uma diversidade nunca antes observada para esse grupo. Em relação aos efeitos de *Wolbachia* sobre a aptidão de *A. petronioi*, foi observado que a bactéria apresenta efeitos negativos sobre a fecundidade desta espécie, embora não influencie a sobrevivência e razão sexual dos indivíduos infectados. Além disso, a taxa de transmissão vertical foi de aproximadamente 85%, um valor alto, considerando os custos associados. Este estudo reforça o fato de que os efeitos da *Wolbachia* são neutros ou deletérios em isópodos terrestres, considerando tanto espécies paleárticas quando neotropicais. Por fim, a filogenia molecular das espécies do gênero Neotropical *Atlantoscia* foi realizada, utilizando-se o gene mitocondrial *COI* (*primers* utilizados no *DNA barcoding*). Foi observada congruência entre as análises moleculares e morfológicas, uma vez que todas as espécies nominais do gênero apresentaram alto suporte nas árvores geradas. Dessa forma, o gene utilizado mostrou-se útil para o seu propósito, podendo ser utilizado em conjunto com as tradicionais descrições e identificações morfológicas de modo a tornar tais análises mais robustas.

Abstract

Wolbachia are known to infect a variety of arthropods and nematodes. In arthropods, the presence of the bacteria has been detected in an impressive amount of insects, crustaceans and chelicerates making *Wolbachia* the most abundant endosymbiont on Earth. The symbiotic relationship between *Wolbachia* and their terrestrial isopod hosts has been known for a long time and is relatively well studied in Europe. However, in South America and Neotropical region, very little is known about the subject. The objectives of this thesis were: to examine the occurrence and prevalence of *Wolbachia* infection in natural populations of terrestrial isopods in South America; to analyze the genetic diversity and phylogenetic relationships of the strains found; to verify the influence of *Wolbachia* on the fitness of *Atlantoscia petronioi*; and to perform the molecular phylogeny of the Neotropical genus *Atlantoscia*. After the first report of *Wolbachia* infection in three species (two native and one introduced) of terrestrial isopods in South America, a broader screening was performed. Animals were collected in 11 Brazilian states, and municipalities of Argentina and Uruguay. The molecular identification of the presence of infection was performed with the gene *16S rDNA*, and positive samples were also tested with the gene *dnaA* and those of MLST (*Multilocus Sequencing Typing - coxA*, *gatB*, *hcpA*, *fbpA*, *ftsZ*). A total of 1172 individuals representing 11 families and 35 species (26 native species and nine introduced species) were tested for the presence of *Wolbachia*. Sixteen species were positive and this is the first report of the infection for eight of those. Despite the low general prevalence of infection, the strains found in the native species showed high diversity, and yet most of them belong to supergroup B, none of the strains are grouped in the *Oni*clado. For the first time, strains of supergroups A and F were recorded in terrestrial isopods, showing a diversity never before seen for the group in question. Regarding the effects of *Wolbachia* on the fitness of *A. petronioi*, was observed that the bacteria have negative effects on the fecundity of this species, although there is no influence on survival and sex ratio of infected individuals. Moreover, the transmission rate was of approximately 85% that is considered high despite the associated costs. This study reinforces the fact that the effects of the infection range from neutral to costly in terrestrial isopods, when considering both Palaearctic and Neotropical species. Finally, the molecular phylogeny of species of the genus *Atlantoscia* was performed using the mitochondrial gene *COI* (primers used in DNA barcoding). We observed congruence between molecular and morphological analyzes, since all nominal species of the genus *Atlantoscia* showed high support in the trees generated. Thus, the gene used was useful for its purpose and can be used together with traditional morphological descriptions and identifications in order to make such analyzes more robust.

Introdução Geral

As bactérias *Wolbachia*

Na evolução bacteriana, a transição de uma existência de vida livre para uma relação estreita com células eucarióticas representa um tema frequente. Certas bactérias simbiontes levaram tais associações ao extremo, abandonando completamente qualquer semelhança com uma fase de vida livre e se replicando apenas dentro do domínio de uma célula hospedeira. Ao longo da história da vida, bactérias obrigatoriamente intracelulares têm atuado como importantes catalisadores evolutivos, estando envolvidas na origem de organelas e na diversificação dos eucariontes. Associações intracelulares atuais compreendem uma gama de relações parasíticas, mutualísticas e comensais nas quais os simbiontes desempenham papéis cruciais na ecologia e fisiologia de seus hospedeiros (Wernegreen, 2005).

A ordem Rickettsiales é composta por diversas espécies de simbiontes obrigatoriamente intracelulares, os quais pertencem à subdivisão alfa de Proteobacteria e apresentam uma ampla variedade de hospedeiros eucariontes (Brenner et al., 1993, Amann et al., 1995). Os principais gêneros de Rickettsiales são *Anaplasma*, *Ehrlichia*, *Orientia*, *Rickettsia* e *Wolbachia* (Renvoisé et al., 2011), sendo este último o objeto de estudo da presente tese.

As bactérias *Wolbachia* são conhecidas por infectar uma variedade de artrópodos e nematoides. Elas foram primeiramente observadas em tecidos reprodutivos do mosquito *Culex pipiens* por Hertig e Wolbach (1924), sendo posteriormente descritas como *Wolbachia pipientis* (Hertig, 1936). Atualmente, uma imensa variedade de linhagens de *Wolbachia* é conhecida, e estas são reunidas em supergrupos (A a N), com base, principalmente, no gene 16S rDNA (Ros et al., 2009). Não existe concordância sobre a validade de todos os supergrupos existentes, sendo que novos supergrupos são frequentemente descobertos (Augustinos et al., 2011). É importante ressaltar que os supergrupos A e B foram os primeiros a serem descritos e incluem a maioria das linhagens que infectam artrópodos (Werren at al., 1995), enquanto que os supergrupos C e D são restritos aos nematoides (Bandi et al., 1998). O supergrupo F, por sua vez, é uma relevante e bem suportada

exceção, sendo o único que abrange linhagens que infectam tanto artrópodes quando nematoides (Casiraghi et al., 2005). De modo a diminuir ambiguidades e facilitar a diferenciação entre as linhagens foi desenvolvido um sistema (MLST - *Multilocus Sequence Typing*) que utiliza cinco genes conservados (*coxA*, *gatB*, *hcpA*, *fbpA*, *ftsZ*) de forma concatenada para tipificar as linhagens de *Wolbachia* de forma inequívoca (Baldo et al., 2006). Essa abordagem tem se mostrado útil para elucidar os caminhos e processos pelos quais tamanha diversidade tem evoluído (Ros et al., 2009).

Como mencionado anteriormente, *Wolbachia* infecta artrópodos e nematoides. No entanto, o tipo de associação da bactéria com cada um desses grupos é essencialmente diferente. Em nematoides, a infecção é conhecida apenas para um subgrupo de filarídeos (Ordem Spirurida, família Filariidae), basicamente todos aqueles que possuem importância médica e veterinária, tais como *Wuchereria bancrofti* (Cobbold, 1877), *Brugia malayi* Brug, 1927, *Onchocerca volvulus* Bickel, 1982 e *Dirofilaria immitis* (Leidy, 1856) (Taylor et al., 2005). Filarídeos e bactérias *Wolbachia* apresentam uma longa história coevolutiva [~100 milhões de anos (Casiraghi et al., 2001)] e uma grande dependência mutualística, tanto que o simbionte se tornou um parceiro essencial para os processos biológicos na vida do nematoide (McNulty et al., 2010). Tratamentos com antibióticos anti-*Wolbachia* são responsáveis por atrasos na muda, taxas de crescimento reduzidas, falha na embriogênese e morte dos filarídeos (Bandi et al., 1999, Hoerauf, et al., 2000). Além disso, períodos de rápido crescimento bacteriano coincidem com o crescimento e desenvolvimento larval e embrionário, o que reforça o papel de *Wolbachia* no fornecimento de nutrientes indispensáveis para esses custosos processos metabólicos (Taylor et al., 2013). Ademais, o desenvolvimento de tratamentos seguros e efetivos para a eliminação dos nematoides adultos através do uso de antibióticos tem se utilizado do fato de que a longevidade dos filarídeos é comprometida quando há a depleção do simbionte (Taylor et al., 2013).

Em artrópodos, nos quais a infecção é facultativa, a presença da bactéria já foi detectada em uma quantidade impressionante de espécies de insetos, crustáceos e quelicerados (Zug & Hammerstein, 2012a), o que faz de *Wolbachia* o mais abundante endossimbionte do planeta Terra (Gerth et al., 2013). Até recentemente acreditava-se que *Wolbachia* era simplesmente um parasito

reprodutivo de artrópodos que se propaga nas populações hospedeiras utilizando a incompatibilidade citoplasmática e a distorção na proporção sexual como mecanismos promotores (Duron et al., 2008). Com o avanço dos estudos ficou claro que a relação entre *Wolbachia* e artrópodos é muito mais complexa do que se imaginava, uma vez que a bactéria é capaz de influenciar a biologia do hospedeiro de maneira que vai muito além do parasitismo reprodutivo (Iturbe-Ormaetxe et al., 2011).

Vários endossimbiontes de artrópodos são conhecidos como sendo parasitos reprodutivos, os quais manipulam a reprodução dos hospedeiros em seu próprio benefício. Os mesmos são transmitidos, predominantemente, de forma vertical, através do citoplasma dos ovos das fêmeas hospedeiras, e não via esperma dos machos (machos representam *dead-ends* para esses microrganismos). Consequentemente, qualquer efeito do simbionte que distorça a razão sexual dos hospedeiros em benefício das fêmeas será seletivamente vantajoso para o endossimbionte (Cordaux et al., 2011). No que diz respeito às bactérias *Wolbachia,* elas não são apenas os mais comuns, mas os únicos parasitos reprodutivos a induzir os quatro tipos mais conhecidos de manipulações reprodutivas: (i) incompatibilidade citoplasmática, (ii) feminização de machos genéticos, (iii) indução da partenogênese, e (iv) morte dos machos (Werren et al., 2008).

Entre os diferentes tipos de manipulação reprodutiva, a incompatibilidade citoplasmática (IC) é o fenótipo mais difundido, ocorrendo em quelicerados (ácaros), crustáceos (isópodos) e insetos (coleópteros, dípteros, hemípteros, himenópteros, lepidópteros e ortópteros) (Werren et al., 2008). Neste fenótipo, a bactéria promove a incompatibilidade entre esperma e ovos em cruzamentos entre machos infectados e fêmeas não infectadas (ou fêmeas que possuem uma linhagem de *Wolbachia* diferente daquela do macho), a qual resulta em alta letalidade de embriões. A IC não induz uma distorção na razão sexual dos hospedeiros, mas favorece a reprodução das fêmeas infectadas e, dessa forma, a transmissão maternal do endossimbionte (Werren et al., 1997). Alguns modelos conceituais foram propostos para explicar a IC (já que os mecanismos exatos não foram totalmente esclarecidos), ambos baseados nas premissas que *Wolbachia* modifica o esperma de modo a causar danos severos no sincronismo e progressão da mitose, e que esta modificação pode ser "recuperada"

por uma fêmea infectada com a mesma linhagem do macho (LePage & Bordenstein, 2013). Os dois principais modelos para a IC são: o modelo chave/fechadura e o modelo do *mistiming*. O primeiro propõe que *Wolbachia* insere certas "fechaduras" no genoma paternal. Uma fêmea infectada com a mesma linhagem da bactéria teria as "chaves" apropriadas para remover essas fechaduras depois da fertilização e recuperar os defeitos mitóticos que possam ocorrer (Poinsot et al., 2003); o segundo modelo, por outro lado, sugere que a IC é resultado de uma falta de sincronismo entre os pró-núcleos materno e paterno, sendo que uma fêmea infectada seria capaz de recuperar essa disparidade fazendo mudanças compensatórias em qualquer um dos pró-núcleos (Ferree & Sullivan, 2006). Em estudo recente, Beckmann & Fallon (2013) propuseram que o processo de indução/recuperação da IC seria resultado de dois genes *operons* (funcionalmente relacionados, contíguos e controlados coordenadamente), onde um gene induz e o outro recupera a IC. Um sistema como este seria muito semelhante à *operons* toxina-antídoto ou "genes de dependência" que são normalmente incorporadas em elementos móveis para aumentar a persistência da segregação (Rankin et al., 2011).

A feminização de machos genéticos é conhecida, principalmente, para crustáceos (isópodos terrestres), mas também ocorre em insetos (lepidópteros e hemípteros) (Werren et al., 2008). Em crustáceos o sexo feminino é o heterogamético (machos são ZZ e fêmeas ZW). Neste grupo, machos genéticos ZZ infectados com *Wolbachia* são convertidos em fêmeas funcionais que, por sua vez, produzem proles com desvio na razão sexual a favor das fêmeas. Um importante resultado dessa evolução é a eliminação do cromossomo sexual feminino W em populações portadores da infecção por *Wolbachia* (Rigaud et al. 1997, Bouchon et al., 1998, Cordaux et al. 2004, Bouchon et al., 2008). Isso acontece porque indivíduos feminizados ZZ produzem fêmeas sem transferir qualquer cromossomo W. Assim, a frequência do cromossomo W diminui a cada geração até que o mesmo seja eventualmente perdido na população. A determinação sexual está, dessa forma, sob o controle de *Wolbachia* em populações infectadas: indivíduos portadores da infecção se desenvolvem como fêmeas enquanto os machos são os indivíduos não infectados (Cordaux et al., 2011). O mecanismo molecular preciso da feminização é desconhecido. No entanto, as evidências indicam que o mesmo

envolve a inibição do desenvolvimento da glândula androgênica durante a diferenciação sexual pós-embrionária (Rigaud et al. 1997, Bouchon et al., 2008). Ambos os sexos, aparentemente, possuem os programas genéticos necessários para a expressão do sexo oposto. O cromossomo W seria um cromossomo Z que carrega um "gene feminino" adicional que inibe a atividade do "gene masculino" localizado no cromossomo Z. Este "gene masculino" controlaria o desenvolvimento da glândula androgênica, órgão responsável pela produção do hormônio androgênico que, por sua vez, provoca a diferenciação sexual masculina e a manutenção dos caracteres sexuais masculinos secundários em adultos (Greve et al., 2004). Em fêmeas genéticas, o "gene feminino" inibe a atividade do "gene masculino", levando à diferenciação sexual feminina. *Wolbachia* seria capaz de agir sobre o "gene masculino" ou sobre uma fase posterior do desenvolvimento sexual, e o resultado final é que a glândula androgênica nunca se diferencia em machos genéticos ZZ infectados pela bactéria (Cordaux et al., 2011). Um efeito de dose também poderia estar envolvido no processo da feminização porque algumas vezes a feminização é incompleta, presumivelmente devido a uma densidade insuficiente de *Wolbachia* para inibir a diferenciação da glândula androgênica, mas suficiente para ativar os receptores do hormônio androgênico em adultos (Rigaud & Juchault, 1998).

A indução da partenogênese foi registrada para insetos haplodiploides (himenópteros e tisanópteros) e quelicerados (ácaros) (Stouthamer, 1997, Huigens & Stouthamer, 2003). Nestes táxons, o sexo é normalmente regulado pela ploidia do embrião: machos se desenvolvem a partir de ovos haploides não fertilizados e fêmeas a partir de ovos diploides fertilizados. *Wolbachia* é capaz de converter os machos não transmissores da infecção em fêmeas transmissoras, fazendo com que ovos não fertilizados se desenvolvam como fêmeas. Esse resultado é alcançado através da duplicação do número de cromossomos em ovos não fertilizados, tornando-os diploides (Huigens & Stouthamer, 2003). Desse modo, fêmeas partenogenéticas infectadas são capazes de produzir prole feminina sem a necessidade da reprodução sexual, fertilização dos ovos, e sem a necessidade de machos (os quais se tornam dispensáveis). Assim como na feminização, o mecanismo molecular subjacente à indução da partenogênese não é totalmente conhecido. Todavia, observações citogenéticas têm mostrado que a partenogênese é induzida através de, pelo menos, três diferentes formas. Em himenópteros,

Wolbachia media a diploidização ao causar perturbações no ciclo celular durante o desenvolvimento embrionário inicial por meio de duas formas: (i) dois conjuntos haploides de cromossomos não se separam durante a anáfase da primeira divisão mitótica, resultando em um núcleo diploide com dois conjuntos idênticos de cromossomos haploides em vez de dois núcleos haploides (Huigens & Stouthamer, 2003), ou (ii) a primeira divisão mitótica é normal, conduzindo a duas células com núcleos haploides, e a diploidia é restaurada através da fusão dos dois núcleos de células, após a conclusão da primeira divisão mitótica (Gottlieb et al., 2002). Um terceiro mecanismo ocorre em ácaros, no qual *Wolbachia* induz a partenogênese através de modificações meióticas em ovos infectados, gerando gametas diploides (Weeks & Breeuwer, 2001).

A morte dos machos já foi registrada para insetos (coleópteros, dípteros, lepidópteros) e quelicerados (pseudoescorpiões). Esse fenótipo leva à distorção da proporção sexual a favor das fêmeas através da morte de progênie masculina [machos se tornam feminizados e morrem durante o desenvolvimento larval (Kageyama & Traut, 2004)]. Ao contrário da feminização e da indução da partenogênese, que consistem na conversão de machos não transmissores de *Wolbachia* em fêmeas transmissoras, a morte dos machos envolve a eliminação do sexo que não transmite verticalmente o endossimbionte. A morte dos machos beneficia as irmãs infectadas através do consumo da prole masculina não desenvolvida (Hurst & Majerus, 1993), da diminuição da intensidade de interações antagônicas entre irmãos e da redução dos níveis de endogamia (Hurst et al., 2003). Como resultado dessa compensação na aptidão, fêmeas infectadas produzem filhas com maior probabilidade de sobrevivência do que fêmeas não infectadas (Hurst et al., 2003). Todavia, essa compensação não seria de uma magnitude elevada (a morte da progênie masculina normalmente aumenta em 10%, ou menos, a probabilidade de sobrevivência das irmãs infectadas). A seleção para endossimbiontes que induzem a morte dos machos não é, dessa forma, muita alta, tanto que a prevalência de infecção na maioria dos hospedeiros é menor do que 40% (Dyson & Hurst, 2004). Além disso, estas prevalências apresentariam grande oscilação temporal e espacial vinculadas às condições ambientais (Jaenike, 2009).

Todos os mecanismos reprodutivos antes mencionados favorecem a propagação de *Wolbachia* nas populações hospedeiras (Werren et al., 2008). Os mesmos dependem da eficiência da transmissão vertical, a qual é o principal mantenedor da infecção ao longo do tempo evolutivo. No entanto, a teoria prediz que, eventualmente, as infecções serão perdidas, provavelmente devido à seleção para a resistência (Koehncke et al., 2009). De modo a assegurar sua pandemia global, as linhagens de *Wolbachia* seriam capazes de ser transmitidas de forma horizontal (entre espécies) com certa regularidade. Mais especificamente, a falta de congruência filogenética entre as linhagens da bactéria e os artrópodos hospedeiros leva a essa conclusão (Baldo et al., 2008, Raychoudhury et al., 2009). No entanto, na maioria dos sistemas *Wolbachia* - hospedeiro, as rotas de transmissão que modelam os padrões de distribuição do endossimbionte permanecem desconhecidas (Gerth et al., 2013).

Segundo Zug et al. (2012b), a transmissão horizontal de *Wolbachia* entre hospedeiros não relacionados (incluindo casos de grande distância filogenética) é necessária para a obtenção dos altos índices de infecção encontrados em muitos clados hospedeiros, algo que não seria possível se considerarmos *Wolbachia* como um parasito transmitido puramente de modo vertical. Os estudos têm demonstrado que as associações ecológicas seriam os meios promotores para a comutação de *Wolbachia* entre espécies taxonomicamente não relacionadas (Stahlhut et al., 2010). Linhagens idênticas ou muito parecidas têm sido encontradas em diferentes espécies hospedeiras que dividem o mesmo nicho ecológico ou possuem interações do tipo presa/predador e hospedeiro/parasita (Kittayapong et al., 2003, Huigens et al., 2004, Kondo et al., 2005, Sintupachee et al., 2006). Assim sendo, a transmissão horizontal de *Wolbachia* envolveria espécies que vivem em íntima associação, e estariam conectadas de maneira trófica e compartilhando o mesmo espaço físico (Stahlhut et al., 2010, Yang et al., 2012). Vale lembrar que, ao contrário dos simbiontes obrigatórios (p. ex., linhagens de *Wolbachia* que infectam filarídeos) que são transmitidos exclusivamente de forma vertical, simbiontes facultativos retêm genomas maiores e com uma grande quantia de elementos móveis de DNA (Newton & Bordenstein, 2011). Consequentemente, eles têm maior oportunidade e habilidade para realizar transmissão horizontal entre indivíduos não aparentados (Duron et al., 2010).

Além das manipulações reprodutivas, é sabido que *Wolbachia* pode, também, influenciar a aptidão de seus hospedeiros. Vários estudos já foram realizados no sentido de determinar os efeitos da infecção, a maioria deles avaliando características como fecundidade/fertilidade e longevidade, sendo que os mesmos demonstram relações que envolvem tanto o parasitismo (Suh et al., 2009, Miller et al., 2010, Sarakatsanou et al., 2011, Vasquez et al. 2011), quanto o comensalismo (Calvitti et al., 2009, Bouwma & Shoemaker, 2011, Friberg et al., 2011) e mutualismo (Zhang et al., 2010, Xie et al., 2011, Zhao et al., 2013, Zhong & Li, 2013). Todavia, os efeitos da infecção de simbiontes verticalmente transmitidos são muito mais complexos do que previamente considerado. Conforme Duran & Hurts (2013), os simbiontes aumentam a aptidão dos hospedeiros mais comumente do que se acreditava o que, de forma indireta, contrabalancearia os custos associados à infecção, facilitando a sua persistência e propagação. Os estudos têm demonstrado que *Wolbachia* seria capaz de proteger seus hospedeiros do ataque de inimigos naturais, interferindo com a multiplicação e replicação de uma ampla gama de patógenos e parasitos, além de impedir a mortalidade induzida por parasitos (Haine, 2008, Brownlie & Johnson, 2009, Wong et al., 2011, Yixin et al., 2013). Assim, é possível que os prejuízos diretos na história de vida dos hospedeiros sejam compensados por benefícios indiretos na sua aptidão.

Uma das mais promissoras áreas de estudo sobre as bactérias *Wolbachia* envolve o desenvolvimento de terapias para o combate de doenças humanas de escala global que possuem artrópodos como vetores. Estes estudos são um exemplo clássico de como a ciência básica pode ser útil para as ciências aplicadas (LePage & Bordenstein, 2013). Uma vez estudada como uma modificação reprodutiva obscura, a incompatibilidade citoplasmática causada por *Wolbachia* é agora o centro das atenções dos esforços para controlar a transmissão de patógenos humanos através de mosquitos vetores. Além disso, estudos têm demonstrado que espécies infectadas com *Wolbachia* têm uma resistência aumentada contra a dengue, Chikungunya (vírus que causa moléstia similar a dengue e que também é transmitido por mosquitos do gênero *Aedes*), febre amarela e vírus do Nilo Ocidental, bem como a malária e bactérias (Moreira et al., 2009, Glaser et al., 2010, Wong et al., 2011, Van den Hurk et al., 2012, Baton et al., 2013). Essa vantagem dupla de *Wolbachia*, de diminuir

a replicação do patógeno e de se propagar via IC em insetos vetores tem implicações diretas para refrear a transmissão de infecções para os seres humanos (LePage & Bordenstein, 2013).

Duas estratégias tiram vantagem deste sistema para reduzir o número de vetores e a competência de transmissão. A primeira visa a liberação de uma grande quantidade de mosquitos infectados por *Wolbachia* (e, por consequência, com depleção de patógenos) os quais poderiam substituir uma população local não infectada, através da IC. Esta estratégia fez progressos impressionantes nos últimos anos, através do Projeto Internacional Eliminação da Dengue (EDP - *Eliminate Dengue Project*) (Hoffmann et al., 2011, Walker, et al. 2011). A segunda estratégia envolve a liberação apenas de machos infectados com linhagens que induzem a IC em populações de vetores não infectados, o que causaria a esterelidade de uma grande fração de fêmeas e reduziria drasticamente o número total de vetores (O'Connor et al., 2012). Esta técnica tem sido empregada com sucesso no controle de pragas agrícolas (Apostolaki et al., 2011), o que revela a capacidade de *Wolbachia* de também ser utilizada como uma alternativa não nociva ao meio ambiente, para o combate de pragas (Zabalou et al. 2004, Bourtzis, 2008, Saridaki & Bourtzis, 2010).

Por fim, embora a presença de *Wolbachia* seja indispensável para os nematoides filarídeos, as relações mutualísticas com artrópodos são muito mais variáveis. Alguns estudos relatam casos nos quais *Wolbachia* confere vantagens aos artrópodos hospedeiros (Zhang et al., 2010, Almeida et al., 2011, Brelsfoard & Dobson, 2011) e, mais interessantes ainda, são os trabalhos que sugerem que a presença do simbionte seria absolutamente necessária para oogênese de alguns indivíduos (Dedeine et al., 2001, Chen et al., 2012). Por mais que os fenótipos induzidos apresentem pouca correlação uns com os outros, uma hipótese interessante é de que eles possam representar vários estágios de um *continuum* de parasitismo para mutualismo entre *Wolbachia* e seus hospedeiros invertebrados. Uma relação de mutualismo ou codependência seria benéfica para ambos os parceiros e poderia ser selecionada ao longo do tempo evolutivo (LePage & Bordenstein, 2013).

***Wolbachia* em isópodos terrestres**

Os isópodos terrestres, subordem Oniscidea, são os crustáceos que obtiveram mais sucesso ao invadir o ambiente terrestre (Bouchon et al., 2008). São conhecidas aproximadamente 3940 espécies de isópodos terrestre (Ahyong et al., 2011), e esses animais são importantes representantes da fauna de solo, participando da formação do mesmo e da reciclagem de nutrientes, além de constituírem fonte alimentar para uma variedade de organismos (Sunderland & Sutton, 1980, Vitt et al., 2000, Van Sluys, 2001).

Há muito tempo sabe-se que os isópodos terrestres possuem microrganismos capazes de transformar machos genéticos em fêmeas funcionais (Legrand & Juchault, 1970), No entanto, a primeira identificação molecular de *Wolbachia* ocorreu somente em 1992 em populações de *Armadillidium vulgare* Latreille, 1802 e *Porcellio dilatatus* Brandt, 1833 (Rousset et al., 1992). Desde então a infecção foi registrada para algumas dezenas de espécies em todo mundo e, especialmente, na Europa (Juchault et al., 1994, Bouchon et al., 1998, Nyirõ et al., 2002, Ben Afia Hatira et al, 2008, Wiwatanaratanabutr et al., 2009, Almerão et al., 2012, Cordaux et al., 2012, Zimmermann et al., 2012). Por mais que os isópodos sejam os principais acometidos [aproximadamente 61% das espécies apresentaria a infecção (Bouchon et al., 2008)], a infecção por *Wolbachia* também é conhecida para outros grupos de crustáceos, tais como anfípodos (Cordaux et al. 2001, Cordaux et al., 2012), ostracodos (Baltanas et al., 2007), cracas (Cordaux et al., 2012) e copépodos (Wiwatanaratanabutr et al., 2013).

Todas as linhagens de *Wolbachia* encontradas até então em isópodos terrestres (e crustáceos em geral) pertencem ao supergrupo B (Bouchon et al., 2008, Cordaux et al., 2012). Além disso, a maioria destas linhagens apresenta grande proximidade filogenética (*Oni*clado), o que sugere uma aquisição ancestral de *Wolbachia* neste grupo (Cordaux et al., 2012). Como a grande maioria dos estudos foi realizada em isópodos terrestres, a revisão que se segue se baseia neste táxon em particular.

Marcadé et al. (1999) observaram que populações de *Porcellionides pruinosus* (Brandt, 1833) infectadas por *Wolbachia* apresentavam pouca ou nenhum variabilidade mitocondrial, o que

poderia estar associado ao modo de transmissão similar utilizado pela bactéria e pela mitocôndria (Hurst & Jiggins, 2005). Segundo os autores, a rápida propagação de um parasito reprodutivo que apresenta alta taxa de prevalência de infecção pode reduzir drasticamente o polimorfismo mitocondrial da população hospedeira. Verne et al. (2012) também constataram que o padrão não usual de polimorfismo mitocondrial encontrado em *A. vulgare* se deve a infecção por *Wolbachia*. Rigaud e Moreau realizaram uma série de trabalhos verificando os efeitos de *Wolbachia* sobre a aptidão de algumas espécies de isópodos terrestres, e concluíram que, de modo geral, a bactéria causa distorções na proporção sexual e prejuízos na fecundidade/fertilidade de fêmeas infectadas e, além disso, os machos preferem copular com as fêmeas não infectadas (Rigaud et al., 1999, Moreau & Rigaud, 2000, Moreau et al., 2001, Rigaud et al., 2001, Rigaud & Moreau, 2004).

A espécie cosmopolita *Armadillidium vulgare* é, certamente, a mais bem estudada no que diz respeito à relação simbiótica com *Wolbachia*. São conhecidas três linhagens da bactéria para esta espécie, com base no gene *wsp*: *w*VulM, *w*VulC e *w*VulP, sendo que a última seria uma versão recombinante das duas primeiras linhagens (Cordaux et al., 2004, Verne et al., 2007). Para o advento desta recombinação seria necessário, em um dos cenários propostos, uma infecção múltipla de *Wolbachia* [(duas ou mais linhagens presentes em um único indivíduo hospedeiro (Verne et al., 2007)]. Ainda que as infecções simples sejam regra para a maioria das associações conhecidas em isópodos terrestres, a presença de infecções múltiplas em *A. vulgare* foi recentemente confirmada (Valette et al., 2013).

No que diz respeito aos modos como *Wolbachia* afeta a imunocompetência dos indivíduos infectados, Braquart-Varnier et al. (2008) testaram indivíduos de *A. vulgare* de uma mesma população e observaram que os indivíduos portadores da linhagem *w*VulC exibiam menor densidade de hemócitos, septicemia mais intensa na hemolinfa e redução do tempo de vida em comparação aos indivíduos portadores de *w*VulM e aqueles não infectados. Sicard et al. (2010) reafirmaram a maior virulência de *w*VulC, demonstrando que além de interferir com parâmetros imunológicos [mais especificamente na atividade da fenoloxidase, envolvida no reconhecimento de patógenos e parasitoides (Liu et al. 2007, Fagutao et al., 2009, Cerenius et al. 2010)], esta linhagem também reduz

a fertilidade de *A. vulgare*. Ainda, em animais infectados, *Wolbachia* coloniza órgãos hematopoiéticos e até um terço dos hemócitos, sendo que o decréscimo na densidade dos hemócitos granulares poderia explicar as deficiências funcionais observadas nesses indivíduos (Chevalier et al., 2011).

Le Clec'h e colaboradores realizaram diversos estudos avaliando os efeitos da transferência horizontal de *Wolbachia*. Após a transferência experimental de *w*VulC de seu hospedeiro nativo, *A. vulgare*, para o aparentado *P. dilatatus dilatatus*, a virulência da infecção aumentou de tal forma que se tornou patogênica. Segundo os autores, a alta virulência esteve associada a uma reação autofágica desregulada no sistema nervoso central dos indivíduos injetados, que além de reduzir a mobilidade e o ganho de peso, culminou com a morte dos mesmos. Este resultado sugere que a tolerância seria a melhor estratégia evolutiva para neutralizar os danos causados pelo parasito, pois a ativação de respostas de resistência poderia não ser efetiva e ainda aumentar a virulência destes parasitos (Le Clec'h et al., 2012).

Le Clec'h et al. (2013a) investigaram a capacidade de oito diferentes linhagens de *Wolbachia* [quatro feminizantes (*w*VulC, *w*VulM, *w*PruIII e *w*Ase) e quatro não feminizantes (*w*Con, *w*Dil, *w*Bre e *w*Pet)] de colonizar diferentes órgãos de *P. dilatatus dilatatus*. Todas estas linhagens foram capazes de invadir a espécie receptora. No entanto, apenas as linhagens feminizantes tiveram impactos negativos sobre a aptidão do novo hospedeiro. Os autores verificaram que além de *w*VulC, as linhagens *w*VulM e *w*PruIII (proveniente de *P. pruinosus*) também causavam a morte *P. d. dilatatus*, sendo que previamente a esta todos os indivíduos injetados apresentavam sintomas similares, tais como paralisia, convulsões, tremor nas pernas e alterações no comportamento de se enterrar. Ademais, foi observado que as linhagens *w*VulC e *w*VulM diferem intrinsecamente nas suas virulências contra *P. dilatatus* e que esta virulência estaria relacionada com a dose de *Wolbachia* e com o tecido utilizado para as injeções (*Wolbachia* transmitida via hemolinfa coloniza os tecidos mais rapidamente e acelera o aparecimento dos sintomas vinculados à infecção). Como *w*VulC alcança maiores concentrações na espécie receptora, é provável que exista uma correlação entre a quantidade de bactéria e os níveis de virulência (Le Clec'h et al., 2014).

Em um interessante experimento, Le Clec'h et al. (2013b) demonstraram que *Wolbachia* pode ser horizontalmente transmitida em isópodos terrestres através do canibalismo. Indivíduos não infectados das espécies *A. vulgare* e *P. d. dilatatus* foram alimentados com indivíduos injuriados de *A. vulgare* portadores da linhagem *w*VulC. Análises de PCR quantitativo e FISH (*Fluorescence in situ Hybridization*) detectaram a presença da bactéria, embora em baixa quantidade, em vários órgãos dos "predadores". Deste modo, os autores sugerem que *Wolbachia* seria capaz de resistir ao processo digestivo e transpor o intestino para em seguida colonizar os órgãos de indivíduos previamente não infectados.

Mais recentemente, Genty et al. (2014) ao utilizarem a técnica de FISH (*Fluorescence in situ Hybridization*), demonstraram que ovários imaturos de indivíduos adultos de *A. vulgare* apresentam muitas áreas não infectadas por *Wolbachia*, com uma proporção de oócitos infectados de 31,5%. Esta proporção aumenta ao longo da maturação do ovário, alcançando 87,6% em oócitos maduros, valor este similar às taxas conhecidas de transmissão vertical da bactéria para a progênie. Conforme os autores, o padrão de infecção sugere que muitos oócitos foram infectados secundariamente, e que a bactéria possivelmente se utilizaria de "reservatórios" presentes em células somáticas ou tecidos para garantir a sua transmissão.

Ainda, Dittmer et al. (2014) realizaram uma investigação quantitativa do padrão de distribuição de *Wolbachia* em diversos tecidos (gônadas, cordão nervoso, hemócitos, intestino e cecos intestinais) de indivíduos de *A. vulgare*, infectados com as três linhagens conhecidas para esta espécie, *w*VulM, *w*VulC e *w*VulP. A bactéria esteve presente em todos os tecidos analisados, independentemente da linhagem considerada. No entanto, as concentrações de *w*VulM foram mais baixas do que aquelas observadas para *w*VulC e *w*VulP. Assim, é provável que os padrões específicos de distribuição sejam (pelo menos em parte) inerentes às diferentes linhagens de *Wolbachia* e/ou o resultado de diferentes interações e coadaptações entre as linhagens da bactéria e *A. vulgare*.

Na região Neotropical foram realizados apenas dois estudos referentes à relação simbiótica entre *Wolbachia* e isópodos terrestres. Almerão et al. (2012) investigaram a presença e diversidade da bactéria nas espécies nativas *Balloniscus sellowii* (Brandt, 1833) e *Balloniscus glaber* Araujo &

Zardo, 1995, e encontraram uma enorme diversidade de linhagens nestes organismos, com base no gene 16S rDNA. Conforme as análises filogenéticas, apesar de fazer parte do supergrupo B, as linhagens presentes em *Balloniscus* não fazem parte do *Oni*clado, indicando que *Wolbachia* seguiu trajetórias evolutivas independentes na América do Sul e na Europa. Resultado similar foi observado por Zimmermann et al. (2012) (primeiro capítulo desta tese) para as espécies nativas *Atlantoscia floridana* (van Name, 1940) e *Circoniscus bezzii* Arcangeli, 1931, corroborando a ideia de que o simbionte pode se propagar diferentemente dentro de cada clado hospedeiro de acordo com a região geográfica (Wiwatanaratanabutr et al., 2009).

Filogenias moleculares em isópodos terrestres

O quarto e último capítulo da presente tese, ao contrário dos outros três, não está diretamente relacionado com a interação dos isópodos terrestres e as bactérias *Wolbachia*, mas é uma consequência da infecção detectada em *Atlantoscia petronioi* Campos-Filho, Contreira & Lopes-Leitzke, 2012. A seguir será apresentado um breve histórico da problemática e a justificativa da elaboração do Capítulo IV. Parte da questão é endereçada no segundo capítulo, o qual aborda os efeitos da infecção por *Wolbachia* sobre a aptidão de *A. petronioi*.

As relações filogenéticas entre as espécies de isópodos terrestres são, ainda, largamente desconhecidas, já que análises robustas começaram a ser realizadas apenas recentemente (Parmakelis et al., 2008). Estas análises, que em um primeiro momento se focaram na filogenia de grupos taxonômicos mais elevados (Wetzer, 2001, 2002, Mattern, 2003), agora visam à resolução das relações entre espécies congenéricas ou entre populações de uma espécie nominal (Rivera et al., 2002, Charfi-Cheikrouha, 2003, Klossa-Kilia et al., 2006, Parmakelis et al., 2008, Poulakakis & Sfenthourakis, 2008, Karasawa & Honda, 2012, Lee et al., 2014).

A discriminação taxonômica de espécies proximamente relacionadas muitas vezes se baseia em características morfológicas sutis, o que dificulta a identificação de rotina (Brökeland & Raupach, 2008). Além disso, se a amostra em questão não possuir representantes do sexo masculino a identificação será impossibilitada, pois a taxonomia em nível de espécie para isópodos terrestres é

primariamente baseada em caracteres sexuais dos machos (Klossa-Kilia, et al., 2005). Tendo em vista que sequências de DNA (especialmente o gene COI, utilizado no DNA *Barcoding*) podem ser usadas na identificação e diferenciação de espécies (Costa et al., 2007), o seu uso conjunto com as análises morfológicas pode ser útil para reduzir os problemas inerentes à utilização isolada das técnicas morfológicas, as quais podem subestimar os verdadeiros níveis de divergência entre populações (Klossa-Kilia, et al., 2005, 2006).

Para a realização do segundo capítulo desta tese foi escolhida uma população de *A. floridana* de Porto Alegre para avaliar os efeitos de *Wolbachia* na aptidão dos organismos infectados. *Atlantoscia floridana* é uma espécie bem estudada e amplamente distribuída (Ferrara & Taiti, 1981, Lemos de Castro, 1985), sendo que uma das principais características que distingue esta espécie é a presença de uma faixa despigmentada, em forma de "U" invertido, no cefalotórax dos animais. No entanto, conforme a diagnose da espécie, nem todos os membros possuem a faixa (Lemos de Castro, 1985, Campos-Filho et al., 2013). Na população em questão, havia tanto indivíduos com a presença (a maioria) e ausência da faixa despigmentada.

Dessa forma, as evidências demonstravam tratar-se uma única espécie, a qual apresentava uma pequena variação na coloração. Ao longo dos experimentos, no entanto, foi observado que apenas indivíduos sem a faixa despigmentada apresentavam a infecção por *Wolbachia*. Logo em seguida, ocorreu a decrição da espécie *A. petronioi* (Campos-Filho et al., 2012), que apesar das grandes semelhanças com *A. floridana* (Campos-Filho et al., 2013), não apresentava a faixa despigmentada no cefalotórax. Ou seja, o que se acreditava ser única espécie eram, na verdade, duas (*A. floridana* e *A. petronioi*), e a presença de *Wolbachia* em apenas um dos morfotipos analisados foi um dos eventos que conduziu a esta conclusão.

Este é um pequeno exemplo de que é necessário cuidado ao se confiar rigorosamente nas identificações morfológicas (por mais bem feito que tenha sido o trabalho dos taxonomistas) e de como o uso de marcadores moleculares seria importante para auxiliar nos processos de descrição e identificação de uma espécie nominal. Considerando que filogenias moleculares nunca foram realizadas para espécies de isópodos terrestres da região Neotropical, nos propomos a realizar a

revisão filogenética do gênero *Atlantoscia* (tema do quarto capítulo), esperando que este estudo seja precursor de tantos outros e que o uso das ferramentas molecular se torne mais recorrente nos trabalhos de taxonomia e sistemática.

Objetivo Geral

O objetivo geral da presente tese foi investigar aspectos da relação simbiótica existente entre as bactérias *Wolbachia* e os isópodos terrestres da América do Sul.

Objetivos específicos

- ❖ Examinar a ocorrência e prevalência de infecção de *Wolbachia* em populações naturais de isópodos terrestres nativos e introduzidos da América do Sul;

- ❖ Identificar a variabilidade e diversidade genética das linhagens e inferir possíveis rotas de transmissão horizontal através da análise das relações filogenéticas das linhagens encontradas;

- ❖ Analisar a influência da bactéria sobre a fecundidade, sobrevivência e proporção sexual de uma espécie nativa de isópodo terrestre naturalmente infectada, *Atlantoscia petronioi*;

- ❖ Determinar as taxas de transmissão vertical da bactéria para a prole desta mesma espécie;

- ❖ Construir a filogenia molecular do gênero *Atlantoscia* utilizando o gene mitocondrial Citocromo c Oxidase Subunidade I - COI.

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Capítulo I

"Detection of *Wolbachia* (Alphaproteobacteria: Rickettsiales) in three species of terrestrial isopods (Crustacea: Isopoda: Oniscidea) in Brazil"

Detection of *Wolbachia* (Alphaproteobacteria: Rickettsiales) in three species of terrestrial isopods (Crustacea: Isopoda: Oniscidea) in Brazil

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Abstract

Terrestrial isopods are widely infected with *Wolbachia*. However, little is known about the presence of bacteria in the Neotropical species. The objective of this study was to test the hypothesis of presence of *Wolbachia* infection in the native species of terrestrial isopods, *Atlantoscia floridana* and *Circoniscus bezzii*, and in the introduced species *Burmoniscus meeusei*.

Key words: *Wolbachia*, terrestrial isopods, 16S rDNA, Brazil.

Wolbachia are Alphaproteobacteria that infect a wide variety of arthropods and nematodes (26). These bacteria use different strategies of symbiosis in their hosts, acting as reproductive parasites in arthropods and as mutualists in nematodes (6). *Wolbachia* has attracted considerable interest, not only because of its pandemic nature (13), but also because of the reproductive phenotypes observed as a consequence of its reproductive parasitism (26). Moreover, the potential of *Wolbachia* as a biocontrol agent has been explored in recent years, with studies that suggest their use in controlling pests and disease vectors (5, 9, 16). Terrestrial isopods are important representatives of the soil fauna, because they take part in soil formation and also in nutrient recycling, in addition to being a food source for a variety of animals (22, 23, 25). This group has developed structural, physiological and behavioral characteristics in order to become fully independent of the aquatic medium for reproduction, which has enabled them to occupy a variety of environments, from the coastal zone to deserts (21).

Terrestrial isopods are widely infected with *Wolbachia*; however, the majority of studies of these associations have been conducted in Europe (8). The first molecular identification of *Wolbachia* was in 1992, in populations of *Armadillidium vulgare* and *Porcellio dilatatus* from France (20). The presence of the bacterium was detected in *Chaetophiloscia elongata* and *Porcellionides pruinosus*, also in France, in 1994 (14). Later, *Wolbachia* was found in more than 14 European species of terrestrial isopods (7) and in *Hyloniscus riparius*, *Trachelipus rathkii* and *T. ratzeburgi*i in Hungary (17). More recently, the infection was detected in 11 species from Tunisia (4) and in two species of *Philoscia* sp. occurring in Thailand (28). Little is known about the presence of bacteria in terrestrial isopods in Brazil, which has a rich isopod fauna of more than 120 species (21).

The objective of the present study was to test the hypothesis of presence of *Wolbachia* infection in the native species *Atlantoscia floridana* and *Circoniscus bezzii*, and in the introduced species *Burmoniscus meeusei*. *Atlantoscia floridana* occurs in the United States, Ascension Island and St. Helena, and from northern Brazil to La Plata in Argentina (2). It is a generalist species in terms of habitat (19), occurring in diverse environments, often in abundance (1, 3). *Circoniscus bezzii* is a little-studied species that occurs in Brazil (in the states of Minas Gerais and Pará) and Paraguay (15).

Burmoniscus meeusei is an introduced species from Asia, with records in England, Hawaii, Brazil and Taiwan (21).

The specimens of *A. floridana* and *B. meeusei* examined were collected in Porto Alegre in Rio Grande do Sul, and the individuals of *C. bezzii* were collected in the municipality of Presidente Olegário in Minas Gerais. The specimens were collected by hand, fixed in absolute ethanol, and transported to the Carcinology Laboratory at Federal University of Rio Grande do Sul, where they were stored in a freezer at -20°C for subsequent DNA extraction. The terrestrial isopods were dissected according to the methodology proposed by Bouchon *et al*. (7). The DNA extractions were performed from reproductive tissue (ovaries of females and utricles of males), part of the nerve cord, and the muscle at the base of the pereiopods, using the Chelex (Bio-Rad) protocol.

The PCR assays to detect the presence of *Wolbachia* were performed under conditions adapted from Bouchon *et al.* (7), targeting the 16S rDNA gene (18). The use of this gene is due to the fact that it has proven to be the most efficient to detect *Wolbachia* in Neotropical terrestrial isopods (data not shown). Part of 16S rDNA gene was amplified using the *Wolbachia* specific primers 99F 5'-TTG TAG CCT GCT ATG GTA TAA CT - 3' and 994R 5' – GAA TAG GTA TGA TTT TCA TGT - 3', which produced fragments of approximately 900 bp (18). The PCRs were carried out in volumes of 25 μl, using 1.0 μl of DNA, 0.16 μl of Platinum® Taq (5 U/μl) (Invitrogen), 2.5 μl of 10X buffer (Invitrogen), 1.66 μl MgCl2 (50 mM) (Invitrogen), 0.5 μl of forward primer (20 μM), 0.5 μl of reverse primer (20 μM), 0.5 μl of dNTPs (10 mM) (Invitrogen) and 18.18 μl of ultrapure water. The amplifications were carried out under the following conditions: 35 cycles (1 min at 95 °C, 1 min at 50.6 °C, 1 min at 72 °C), including an initial denaturation step of 95 °C for 2 min and a final extension step of 72 °C for 5 min. As a positive control for the PCR reaction, DNA extracted from an individual of the terrestrial isopod *Balloniscus glaber* was used, in which infection by *Wolbachia* was previously detected (unpublished data). PCRs were confirmed by electrophoresis in 1% agarose gel. Possible failures in the amplifications with the 16S rDNA primers could occur for the following reasons: (i) absence of *Wolbachia*; (ii) a flaw in the DNA extraction process; and/or (iii) an incorrect concentration of DNA solution (27). In order to control the last two possibilities, we tested samples assumed to be

negative, with primers of subunit I of the Cytochrome Oxidase mitochondrial gene (*COI*) (11). Samples that generated a product of the expected size were considered to be truly negative for the presence of *Wolbachia*. The amplified DNA fragments of positive samples were sent to the company MACROGEN Advancing through Genomics for purification and sequencing. This company uses the BigDye™ Terminator protocol, and sequencing was conducted on a 3730xl DNA analyzer.

The resulted sequences were compared by the Blastn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with sequences deposited in the GenBank database. All sequences were subsequently submitted in the Genbank-EMBL database (http://www.ncbi.nlm.nih.gov/) under accession numbers: JF799948, JF799949, JF799950 and JF799951. Alignment of sequences was performed using the BioEdit program (12). The *Wolbachia* strain names were based on the nomenclature system proposed by Zhou *et al.* (29) and specified by Charlat *et al.* (10). Each strain's name is defined by w (in italics) denoting *Wolbachia*. This is followed by three letters coming from the first three letters of the species name. Multiple strains present in a given species are distinguished by numbers added at the end.

Positive samples were detected in the three species *A. floridana*, *B. meeusei* and *C. bezzii*, representing the first record of *Wolbachia* infection in these species. The infection was observed in 30 individuals: 25 females of *A. floridana*, 2 females of *B. meeusei* and 3 females of *C. bezzii* (Table 1). A greater number of individuals of *A. floridana* were tested because this species usually occurs in abundance in nature (1), which facilitates their collection. However, *B. meeusei* and *C. bezzi* are difficult to collect. In addition, this is the first record of the presence of *B. meeusei* in the state of Rio Grande do Sul; until now, this species has been known only from Santa Catarina. A single 16S rDNA sequence from *Wolbachia* was identified for *A. floridana* (*w*Flo) and *B. meeusei* (*w*Mee). For *C. bezzii*, two very different 16S rDNA sequences (*w*Bez1 and *w*Bez2) were found in two different individuals (Figure 1). Thus, multiple infections were not detected. Natural multiple infections in a single host have never been demonstrated in terrestrial isopods species (24), and this work supports this statement.

This study reports one of the first records of *Wolbachia* infection in species of terrestrial isopods in Brazil, in particular in the native species *A. floridana* and *C. bezzii* and the introduced species *B. meeusei*. Although these bacteria are widely studied, very few investigations of their interactions with terrestrial isopods and other organisms have been carried out in the Neotropical region. It is hoped that further studies will be undertaken to expand knowledge of the *Wolbachia* bacteria and their Neotropical hosts.

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Table 1: Total of individuals of each species tested for the presence of *Wolbachia*. FT: females tested;

MT: males tested; FI: females infected; MI: males infected; RS: Rio Grande do Sul; MG: Minas Gerais.

Species	FT	MT	FI	MI	Locality	Geographical Coordinates
Atlantoscia floridana	45	3	25	0	Porto Alegre/RS	30°04'76''S/51°07'28''W
Burmoniscus meeusei	2	0	2	0	Porto Alegre/RS	30°04'49''S/51°07'31''W
Circoniscus bezzii	7	0	3	0	Presidente Olegário/MG	18°24'02''S/46°25'50''W

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                  10        20        30        40        50        60        70        80
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
wFlo     ATCTACCTAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCCTACGGGGGAAAAATTTATTGCTATTAG
wMee     ................................................................................
wBez1    ................................................................................
wBez2    ...A............................................................................

                  110       120       130       140       150       160       170       180
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
wFlo     TTAGCTAGTTGGTGGAGTAATAGCCTACCAAGGCAATGATCTATAGCTGATCTGAGAGGATGATCAGCCACACTGGAACTGAGA
wMee     ....T........AG....AG..T......A..................................................
wBez1    ................................................................................
wBez2    ................................................................................

                  210       220       230       240       250       260       270       280
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
wFlo     ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCATGAGTGAAGAAGGCCTTTGGGTT
wMee     ..............................................................................C.....
wBez1    ..............................................G.......................C.........
wBez2    ................................................................................

                  310       320       330       340       350       360       370       380
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
wFlo     GAGGAAGATAATGACGGTACTCACAGAAGAAGTCCTGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGAGGGCTAGCGTT
wMee     ................................................................................
wBez1    .............A..................................................................
wBez2    ................................................................................

                  410       420       430       440       450       460       470       480
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
wFlo     CGTAAAGGGCGCGTAGGCTGATTAATAAGTTAAAAGTGAAATCTCGAGGCTTAACCTTGGAATTGCTTTTAAAACTATTAATCT
wMee     ................G.G...G.................C.A.....C...........C.............GC......
wBez1    ................G...............................C...............................
wBez2    ................G...G...................C...............C.............GC...C..

                  510       520       530       540       550       560       570       580
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
wFlo     TAGAGGAATTCCTGATGTAGAGGTAAAATTCGTAAATATTAGGAGGAACACCAGTGGCGAAGGCGTCTATCTGGTTCAAATCTG
wMee     ..............AG.........G......................................................
wBez1    ................................................................................
wBez2    ...........AG........GG....................................G.............
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                  610       620       630       640       650       660       670       680
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
wFlo     GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAATGTTAAATATGGGAAGTTTACTTTCTGTA
wMee     ................................................................................
wBez1    .............T..................................................................
wBez2    ................................................................................

                  710       720       730       740       750       760       770       780
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
wFlo     AAACATTCCGCCTGGGGACTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGT
wMee     ................................................................................
wBez1    ................................................................................
wBez2    ................................................................................

                  810       820
         ....|....|....|....|....|...
wFlo     CGAAAAACCTTACCACTTCTTGAC-TGA
wMee     ................C......A...
wBez1    .......................A...
wBez2    ................C......A...
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Figure 1: Alignment of *Wolbachia* 16S rDNA sequences found in *A. floridana* (*w*Flo), *B. meeusei* (*w*Mee) and *C. bezzii* (*w*Bez1 and *w*Bez2).

Capítulo II

"Effects of *Wolbachia* on the fitness of a Neotropical terrestrial isopod"

Artigo submetido para a revista *Journal of Invertebrate Pathology* (ISSN: 0022-2011)

Effects of *Wolbachia* on the fitness of a Neotropical terrestrial isopod

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Abstract

Wolbachia is a diverse group of intracellular bacteria that infect a variety of arthropods and some groups of nematodes. The objectives of the study were to evaluate *Wolbachia* effects on fecundity, survival and sex ratio of the Neotropical terrestrial isopod *Atlantoscia petronioi*, as well as to determine the vertical transmission rates of *Wolbachia* to the offspring. We also intend to use molecular markers to corroborate the species level of *A. petronioi*, which until recently was believed to be a variant form within *A. floridana.* Despite the high morphological similarity the phylogenetic analyzes support the separation of these species and only *A. petronioi* individuals are naturally infected. *Wolbachia* presents negative effects on the fecundity of *A. petronioi*, whereas it is not affecting survival and sex ratio. The vertical transmission rates in this study were 85%. This was the first study to evaluate effects of *Wolbachia* in Neotropical terrestrial isopods and it corroborates the fact that effects of the infection range from neutral to costly in this group.

Key-words: Neotropical, terrestrial isopods, *Wolbachia*, fitness effects, vertical transmission, genetic diversity.

1. Introdution

Wolbachia is a diverse group of intracellular bacteria that infect a variety of arthropods and some groups of nematodes. The frequencies of infection in terrestrial arthropods have been recently estimated at around 40% (Zug and Hammerstein, 2012), which would make this bacteria the most abundant endosymbiont in the planet. *Wolbachia* transmission is associated with the evolution of several reproductive phenotypes, including the feminization of genetic males, the cytoplasmic incompatibility, the induction of parthenogenesis and death of genetic males (Werren et al., 2008). Although strategy may vary, the multiple mechanisms through which *Wolbachia* manipulates the hosts reproduction are similar in that they bring reproductive advantages to infected females over the non-infected ones (Xie et al., 2011). Besides the reproductive effects, *Wolbachia* can also influence its hosts fitness. Studies show that the *Wolbachia* - host interaction varies along a continuum of parasitism to mutualism (Werren et al., 2008), in which fitness costs or benefits may either promote the spread of the infection or make it difficult (Dobson et al., 2002).

Terrestrial isopods are highly diverse group of over 3,900 species (Ahyong et al., 2011) including important representatives of the soil fauna, accounting for a great part of it and influencing its dynamics (Quadros et al., 2009). The symbiotic relationship between the terrestrial isopods and *Wolbachia* has been mainly studied in Europe and, according to estimates, about 61% of the species are infected (Bouchon et al., 2008). All currently strains of *Wolbachia* known in terrestrial isopods belong to the Supergroup B and most strains present great phylogenetic similarity (Cordaux et al., 2012). Few studies assess the influence of *Wolbachia* over aspects of the life history of terrestrial isopods. The studies carried on so far demonstrated neutral or deleterious effects resulting from the infection, both for individuals naturally infected and for those who were artificially infected with *Wolbachia* (Braquart-Varnier et al., 2008; Le Clec'h et al., 2012, 2014; Rigaud et al., 1999, 2001).

In South America, studies concerning the symbiotic relationship *Wolbachia* – terrestrial isopods are still incipient. Those already conducted aimed at an analysis of presence, prevalence of infection and genetic diversity of the *Wolbachia* strains in species of the native fauna (Almerão et al.,

2012; Zimmermann et al., 2012), meanwhile, studies that evaluate the bacteria effects on the fitness of infected individuals have never been conducted.

The Neotropical genus *Atlantoscia*, family Philosciidae, includes five species, *A. floridana* (Van Name, 1940), *A. rubromarginata* Araujo and Leitikow, 1999, *A. petronioi* Campos-Filho, Contreira and Lopes-Leitzke, 2012, *A. sulcata* Campos-Filho, Lisboa and Araujo, 2013 and *A. ituberasensis* Campos-Filho, Lisboa and Araujo, 2013. Most species of the genus have been described only recently and due to the great biodiversity of the Neotropical region it is believed that many more remain to be described. However, the great similarity of species hinders their morphological identification, even more that such identification is based primarily on sexual characters of males (Campos-Filho et al., 2013). Furthermore, molecular analyzes that could aid in the differentiation of species have never been performed for terrestrial isopods in the Neotropics. *Atlantoscia floridana* is the best studied species (Araujo and Bond-Buckup, 2004, 2005; Araujo et al., 2004a, 2004b), and the only one with wide distribution [Brazil (coastal states, Trinidad and Abrolhos islands), Argentina, Ascension and Santa Helena islands, and also Florida in the United States of America (Ferrara and Taiti, 1981; Lemos de Castro, 1985)]. Life history and ecological aspects are unknown for the other species in the genus.

The objectives of the current study were to evaluate *Wolbachia* effects on fecundity, survival and sex ratio of the host *Atlantoscia petronioi*, as well as to determine the vertical transmission rates of the bacteria in this species. We also intend to use, for the first time in South America, molecular markers to corroborate the taxonomic entity *A. petronioi*, which until recently was believed to be a variation within *A. floridana* (Campos-Filho et al., 2012; Lemos de Castro, 1985; Zimmermann et al., 2012).

2. Materials and methods

2.1 Collection of animals

Atlantoscia petronioi individuals were collected from a population of Porto Alegre, Rio Grande do Sul, Brazil, located in the campus of the Federal University of Rio Grande do Sul

(30°04'00''S/51°07'19''W). Inasmuch as *Wolbachia* infection prevalence is high in this population (about 80%, BLZ personal observation), individuals from the species *Atlantoscia floridana* were used for the comparative analysis since they are morphologically similar (Figure 1) and occur in syntopy with *A. petronioi*, and are not infected by *Wolbachia* (BLZ personal observation). The animals from both species were collected from January 2011 to July 2012, taken to the Laboratory of Carcinology of the Federal University of Rio Grande do Sul and kept in containers with leaves and field soil, under laboratory controlled conditions (temperature of 20 °C and photoperiod of 12: 12). Part of the material was later transferred to the Laboratory of Ecology, Evolution and Symbiosis, University of Poitiers, for additional experiments (see below).

2.2 Extraction, PCR and sequencing

DNA from terrestrial isopods was extracted using the Chelex protocol (Bio Rad). Eggs, embryos and mancas extraction were performed with the extraction kit PureLink Genomic DNA Kits (K1820-01). Extraction success was verified by amplification with the universal primers of the gene Cytochrome Oxidase I (COI – Folmer, 1994). Furthermore, the COI gene was used to verify the existent of genetic diversity between the species *A. floridana* and *A. petronioi* in the population of Porto Alegre. For the molecular analysis, specimens of *Atlantoscia* sp. collected in Porto Alegre (30°11'25''S/51°10'31''W) and not infected with *Wolbachia* (BLZ personal observation) were also utilized. *Burmoniscus meeusei* (family Philosciidae) was used as outgroup.

Bacterial 16S rDNA gene (Primers 99F 5'- TTG TAG CCT GCT ATG GTA TAA CT - 3' and 994R 5' – GAA TAG GTA TGA TTT TCA TGT - 3', O'Neill et al., 1992) was used in order to detect *Wolbachia*. All the reactions were carried out in volumes of 25 µl, using 1.0 µl of DNA, 0.16 µl of Taq Platinum (5U/µl), 2.5 µl of 10X buffer, 1.66 µl of $MgCl_2$ (50 mM), 0.5 µl of forward primer (20 µM), 0.5 µl of reverse primer (20 µM), 0.5 µl of dNTPs (10 mM) and 18.18 µl of ultrapure water. For the COI amplifications, the following settings were used: 35 cycles (50 s to 95 °C, 50 s to 57 °C and 50 s to 72 °C) with initial denaturation at 95 °C for 2 min and final extension at 72 °C for 5 min. For the 16S rDNA gene the settings were as follows: 35 cycles (1 min at 95 °C, 1 min at 50.6 °C and 1 min at 72 °C)

with initial denaturation at 95 °C for 2 min and final extension at 72 °C for 5 min. Sequences were obtained using BigDye technology by Macrogen Inc., Korea and deposited in the GenBank-EMBL database (http://www.ncbi.nlm.nih.gov/).

2.3 Phylogenetic analyzes

The bacterial 16S rDNA and mitochondrial COI sequences were aligned with ClustalW (Thompson et al., 1994) and alignments were visually inspected by using the BioEdit 7.1 (Hall, 1999). The alignment of the COI data set (632 nucleotides) was verified against published COI sequences of other isopod species available in GenBank. The COI sequences produced in the present study were unambiguously aligned to the retrieved COI sequences, whereas no gaps and/or stop codons were present the mtDNA sequence data set. Therefore, the authenticity of the produced mtDNA sequences was verified. Nucleotide divergence within and between species were estimated using MEGA 6.0 (Tamura et al., 2013) and the Kimura two-parameter model (Kimura, 1980). jModelTest 2.1.3 (Darriba et al., 2012) was used to select the best-fitting substitution model for the COI alignment according to the Akaike Information Criterion (AIC - Akaike, 1974). Phylogenetic analyses were conducted using Maximum Likelihood (ML) method with 1,000 bootstrap replicates, using TrNe+I as a model of nucleotide substitution, implemented in MEGA 6.0 (Tamura et al., 2013).

2.4 Effects of *Wolbachia*

For the fecundity analysis, ovigerous females from *A. petronioi* and *A. floridana* were individualized, measured (cephalothorax width) and had their offspring (eggs, embryos and mancas) counted (Araujo and Bond-Buckup, 2005). Statistical analysis consisted of the comparison, through an analysis of covariance, of the linear regressions obtained from the infected females (*A. petronioi*) *versus* the non-infected ones (*A. floridana*). Differences in the average size and fecundity of the analyzed species were calculated with the Mann-Whitney test. For the survival analysis, seventeen infected ovigerous females from *A. petronioi* and 17 non-infected ones from *A. floridana* were individualized and observed daily until the release of mancas. The offspring was followed during 50

days, a period in which the individual mortality was observed. The survival curves obtained for the infected and non-infected female's offspring were compared with the Log-Rank test.

In order to verify whether the offspring of females infected and non-infected with *Wolbachia* presented deviation in the sex ratio, 20 infected ovigerous females of *A. petronioi* and 17 non-infected ovigerous females of *A. floridana* were individualized and, after the release of mancas, these mancas were monitored until sexual differentiation was evident (development of male genitalia and the female genital pore, according to Araujo et al., 2004b). The chi square test was used for comparisons between the sex ratios. All these procedures were performed using Bioestat 5.0 software (Ayres et al., 2007). For the analysis of vertical transmission, the offspring of 30 ovigerous females from *A. petronioi* infected with *Wolbachia* were tested, individually, in order to determine the proportion of individuals that inherited the infection.

2.5 Transfection experiments

Fifteen *A. floridana* females from the naturally non-infected population of Porto Alegre received *Wolbachia* injections in order to verify whether the bacteria could infect this species and be passed on to the next generations. Suspensions were prepared with the ovaries of five *A. petronioi* infected females. The ovaries were collected and crushed into 0.5 ml of Ringer solution. The resulting suspension was filtered through a 1.2 mm pore membrane, and 1 ml of each filtrate was injected into a small hole pierced in each individual cuticle, using a thin glass needle, into the general cavity, at the posterior part of the animals. Five *A. floridana* females received injections only with the Ringer solution and were used as control group to assess the effect of the manipulation of the animals. The injected *A. floridana* females were kept in plastic containers with leaves and soil *ad libitum* and were later put to breeding with males of the same species. These experiments were carried out in the Laboratory of Ecology, Evolution and Symbiosis, University of Poitiers.

3. Results and discussion

Atlantoscia petronioi and A. floridana are very similar species in terms of morphology (Campos-Filho et al., 2013). One of the most commonly used characters to identify A. floridana is its color pattern, more specifically the presence of an inverted U-shaped, depigmented and notable stripe on its cephalothorax. Some specimens are, however, described as not exhibiting such pattern (Campos-Filho et al., 2013; Lemos de Castro, 1985). Individuals of A. petronioi do not present the depigmented stripe on the cephalothorax, but the general morphological similarity to A. floridana and the syntopic occurrence of these two species in the study area led us to believe, at the beginning of the experiments, that all individuals examined belonged to A. floridana (Zimmermann et al., 2012).

Only after the description of the A. petronioi in Dec-2012 and the finding that only specimens without the depigmented stripe on the cephalothorax presented infection with Wolbachia (all infected individuals presented the same 16S rDNA strain of Wolbachia, the same observed by Zimmermann et al., 2012), it was possible to confirm that there were, in fact, two distinct species. As stated above, taxonomy in species level for terrestrial isopods has been based, mainly, in a few sexual characters of the males, although analyzes with molecular markers have indicated that the definition of species based on morphology may underestimate the true levels of divergence between populations (Klossa-Kilia, et al., 2005, 2006). Moreover, in many groups of genera or species, morphological characters do not provide by itself a clear taxonomic resolution, so that changes in interpretation of nominal species are constantly reported in the literature (Schmalfuss, 2003).

Although preliminary, this study presents the first molecular phylogeny developed for terrestrial isopods in the Neotropical region. For each species analyzed, two haplotypes were found considering the COI gene. The average genetic distance within species was 0.3% (±0.002) in A. petronioi, 1% (±0.004) in A. floridana and 4.4% (±0.008) in Atlantoscia sp. The average genetic distance between A. floridana and Atlantoscia sp. was 14.7% (±0.015), between A. floridana and A. petronioi was 14.9% (±0.015) and between A. petronioi and Atlantoscia sp. was 18.2% (±0.017). In addition to that, the COI gene ML tree demonstrated well supported clades for each of the species (Figure 2).

The genetic divergence observed between the species *A. petronioi* and *A. floridana,* of nearly 15%, evidence that despite the great morphological similarity, the species present a considerable genetic distance. According to Costa et al. (2007) the genetic variation of the sequences observed in the barcode region of the COI gene is very effective to discriminate species of crustaceans and the average value of divergence to separate congeneric species is 17.16%. Other well-defined species of isopods are reported to be separated by genetic distances (estimated from COI and other gene segments like 12S and 16S rDNA) in the range of 7.2–28% (Baratti et al., 2004; Charfi-Cheikrouha, 2003; McGaughran et al., 2006; Parmakelis et al., 2008; Rivera et al., 2002; Xavier et al., 2011, 2012). Furthermore, the low intraspecific diversity observed in *A. petronioi* compared to other species may be the result of *Wolbachia* infection, since a rapid spread of a reproductive parasite that has high prevalence of infection can dramatically reduce the mitochondrial polymorphism of the host population (Behura et al., 2001; Marcadé et al., 1999).

All *A. floridana* females died in up to three weeks after having received the *Wolbachia* injections, unlike those which received only the Ringer injections. This result suggests a lack of coadaptation between symbiont and host. There are two plausible and non-exclusive explanations for the high level of virulence caused by a symbiont when infecting a new host: either poorly adapted symbionts may explore their host excessively, multiplying quickly or in some unexpected organ; or poorly adapted hosts may respond to the presence of an unknown symbiont by triggering inefficient and costly immune defenses (Lipsitch and Moxon, 1997).

Bouchon et al. (1998), in an experiment with terrestrial isopods, observed that the feminizing *Wolbachia* from *Armadillidium* species killed the recipient *Porcellio dilatatus* males after about 30-60 days. According to the authors, the factor responsible for lethality in this interaction is unknown, but Juchault et al. (1974) observed a massive symbiont proliferation, followed by necrosis of the nervous tissues. Bouchon et al. (1998) also observed that death occurred after a sort of paralysis. Thus, interaction between the bacterium and the nervous system is probably involved in this mortality. More recently, Le Clec'h et al. (2012) verified that when a strain of *Wolbachia* was transferred from a native host (*Armadilliduim vulgare*) to a new host (*Porcellio dilatatus dilatatus*),

the increase in virulence was linked to an unregulated autophagic reaction in the central nervous system of the individuals that received the infection, which ultimately caused the death of such individuals.

Since they belong to the same genus, are morphologically similar and live in syntopy (they are subject to the same environmental pressures), either a similar or a not very dissonant pattern would be expected regarding the aspects of *A. floridana* and *A. petronioi* life histories. To check the effects of *Wolbachia* infection, 330 ovigerous females were analyzed, 206 non-infected ones of the *A. floridana* species and 124 naturally infected ones of the *A. petronioi* species. The average size of non-infected females was 1.31 ± 0.13 mm and the average fecundity was 12.28 ± 5.06 eggs. As for the average size of infected females, it was 1.41 ± 0.09 mm and the average fecundity was 9.6 ± 2.49 eggs. In spite of *A. petronioi* females being significantly larger than *A. floridana* ones (Z (U)= 7.15, $p<0.01$), the latter were significantly more fecund (Z (U)= 4.32, $p<0.01$). The coefficient of determination of linear regression (size *versus* fecundity) obtained for non-infected *A. floridana* females was $R^2= 0.62$ (F = 335.5, $p < 0.01$). For *A. petronioi* this value was $R^2= 0.26$ (F = 44.08, $p < 0.01$) (Figure 3). The covariance analysis showed a significant difference between the regressions obtained for infected and non-infected females ($F_{1.33}$ slope= 22.36, $p<0.01$; $F_{1.33}$ intercept= 231.65, $p<0.01$).

Despite having a larger average size, infected females of *A. petronioi* presented lesser average fecundity than *A. floridana*, and besides, the coefficient of determination found for *A. petronioi* was much lower than expected. The positive correlation between size/mass of ovigerous females and their fecundity is a well-established reproductive pattern, one that has been registered for many crustacean species (Sutton et al., 1984). Beyond that, *A. floridana* showed a set of characteristics corresponding to those of the r-strategists: a shorter life span, faster development, earlier reproduction and a reproductive allocation to maximize brood size (Quadros et al., 2009), and it is likely that *A. petronioi* share these same characteristics. In this way, it is probable that the infection by *Wolbachia* is causing a direct impairment of *A. petronioi* fecundity.

After the 50-day period, the offspring of *A. petronioi* females presented a mortality rate of 72% (122 out of 170 individuals). The offspring of *A. floridana* females, on the other hand, presented a mortality rate of 68% (147 out of 216 individuals). There was no significant difference in the survival curves of the two groups tested (Log-Rank test, p = 0.48) (Figure 4). The high mortality levels are in accordance with the literature, since in *A. floridana* most of the individuals die in the first stages of life, with only 1% of the population reaching one year old (Araujo and Bond-Buckup, 2005; Quadros et al., 2009). Thereby, *Wolbachia* would not be influencing the survival in the first stages of life of infected individuals.

Regarding the sex ratio, the offspring of *A. floridana* females non-infected with *Wolbachia* presented deviation in favor of females by 1.4:1 (p>0.05). According Araujo and Bond-Buckup (2005), males are clearly more numerous in adulthood when considering the operational sex ratio of *A. floridana*, but the sex ratio in the juveniles showed a different pattern, favoring females (1:2.2). Offspring of *A. petronioi* females infected with *Wolbachia*, in turn, presented deviation in favor of males (1.3:1). Nonetheless, there was no significant difference concerning the expected proportion of 1:1 (p > 0.05).

In spite of the most diverse effects on the fitness of infected individuals having been registered for *Wolbachia* (Werren et al., 2008), in terrestrial isopods only relations involving commensalism and parasitism are known. Rigaud et al. (1999) observed deviation in the sex ratio in favor of females, and fecundity reduction in infected females of *Oniscus asellus*, although a difference in fertility between infected and non-infected females has not been observed. Rigaud et al. (2001) demonstrated fertility reduction in females of *A. vulgare*, *P. scaber* and *O. osellus* after they had received *Wolbachia* injections from their infected conspecifics. Rigaud and Moreau (2004) observed that the sperm depletion, caused by multiple copulations, affects only the fertility of infected females of *A. vulgare*, which would be related to the fact that the males prefer to copulate with non-infected females. Still regarding *A. vulgare*, it has been demonstrated that the *w*VulC strain is the most virulent and reduces survival, fertility, number of hemocytes and immunological parameters of the infected individuals (Braquart-Varnier et al., 2008; Sicard et al., 2010). Lastly, Le

Clec'h et al. (2012) verified that, when receiving injections with the *w*VulC strain, *P. d. dilatatus* specimen showed a decrease in weight gain and mobility, whereas in the reciprocal experiment no significant effects of *Wolbachia* were detected. Moreover, there is a potential link between the bacterial titers and the levels of virulence and *Wolbachia* transinfected via hemolymph colonized the body of the recipient host more quickly and caused accelerated symptoms compared to *Wolbachia* introduced via a crushed ovaries suspension (Le Clec'h et al. 2014).

Regarding the vertical transmission, of the 273 offspring individuals tested for the presence of *Wolbachia*, 232 showed the infection. Thus, the vertical transmission rate in *A. petronioi* was 84.98%. This vertical transmission is considerably high, since in nature there are many factors which prevent a perfect transmission of the infection (Clancy and Hoffmann, 1998; Merçot and Poinsot, 1998). High levels of vertical transmission, of 94.1%, were also observed for naturally infected individuals of *Cylisticus convexus* (Moret et al., 2001). The success of the bacteria is critically dependent on the efficiency of the vertical transmission in nature, since this manner of transmission is the principal maintainer of the infection throughout evolutive time (Koehncke et al., 2009).

According to a review done by Duron and Hurts (2013), the vertically transmitted effects of the infection of symbionts are much more complex than previously known. Symbionts may increase host fitness more commonly than usually assumed which, in an indirect way, would counterbalance the costs associated with the infection and allow the propagation and persistence of the infection. *Wolbachia* may be capable of protecting its hosts from the attack of natural enemies by interfering with multiplying and replication of a great scale of pathogens and parasites, besides preventing parasite induced mortality (Brownlie and Johnson, 2009; Haine, 2008). Thus, it is likely that the direct impairment to the host life history is compensated by indirect benefits to the fitness of these animals.

In conclusion, *Wolbachia* effects range from neutral to costly in terrestrial isopods, considering both Palearctic and Neotropical species. *Wolbachia* presents negative effects on the fecundity of *A. petronioi* and high values of vertical transmission, whereas it is not affecting survival and sex ratio of the infected individuals. Nonetheless, studies to evaluate possible indirect benefits of

the infection have never been performed in this group, and such studies could explain the reason behind the maintenance of the infection in the host species despite the direct costs associated. More studies are needed in order clarify the respective roles of the direct and indirect effects of *Wolbachia* on the dynamics and persistence of the infection in terrestrial isopods.

4. Acknowledgments

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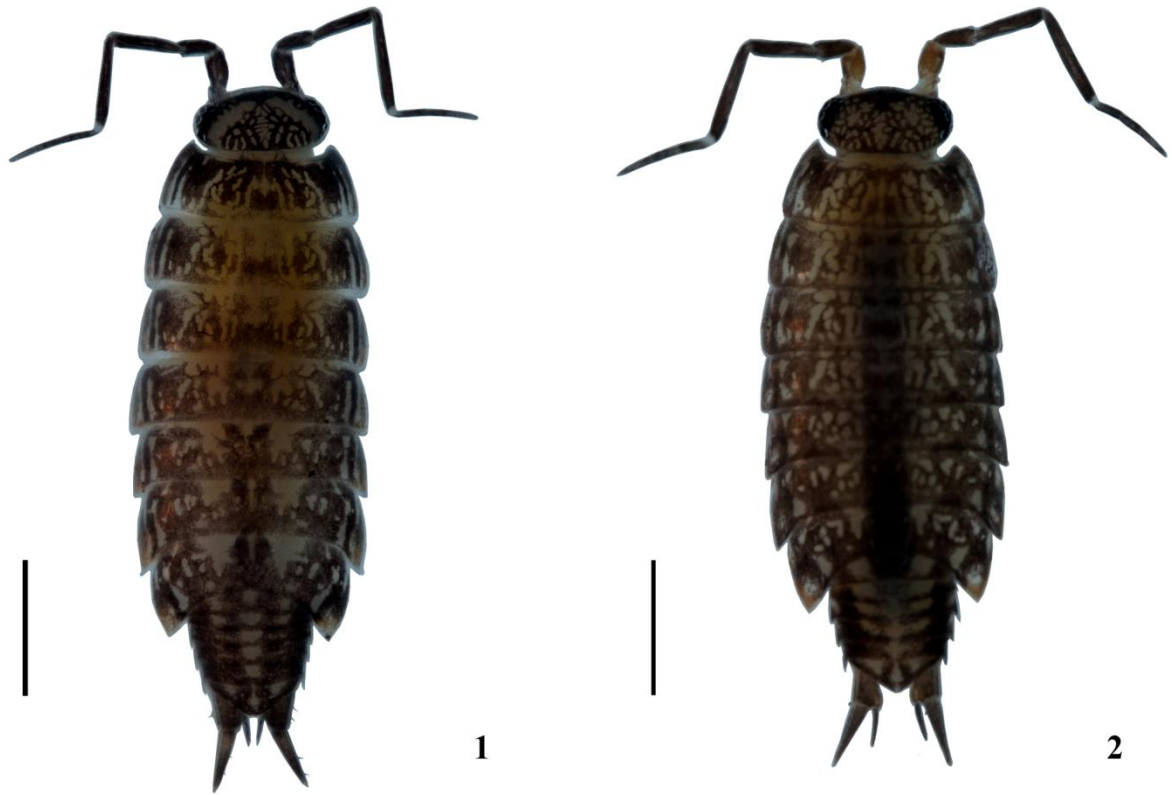
Figure 1: *Atlantoscia floridana* (1) and *Atlantoscia petronioi* (2), collected in Porto Alegre, Brazil. Scale bar represents 1 mm.
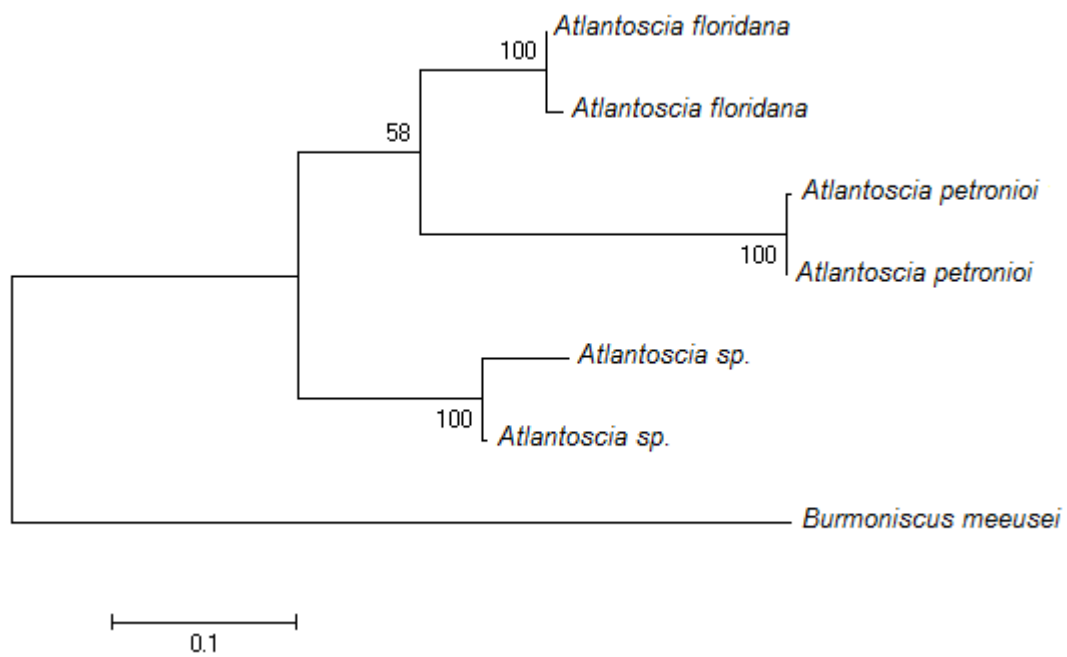
Figure 2: Maximum-likelihood tree obtained for the COI gene. Values of nodes correspond to bootstrap support. GenBank accession numbers: KJ509606-KJ509612.
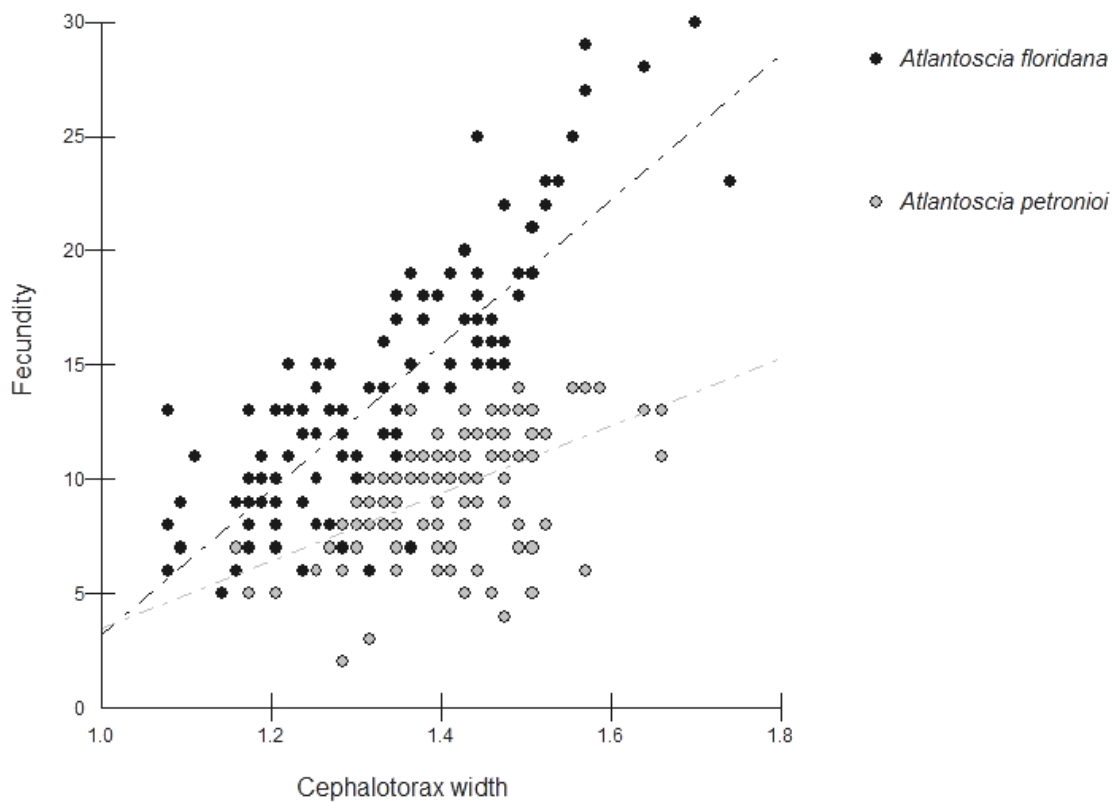
Figure 3: Linear regressions, cephalotorax width (mm) *versus* fecundity (number of eggs), obtained

for *Atlantoscia petronioi* (*Wolbachia* positive) and *Atlantoscia floridana* (*Wolbachia* negative).
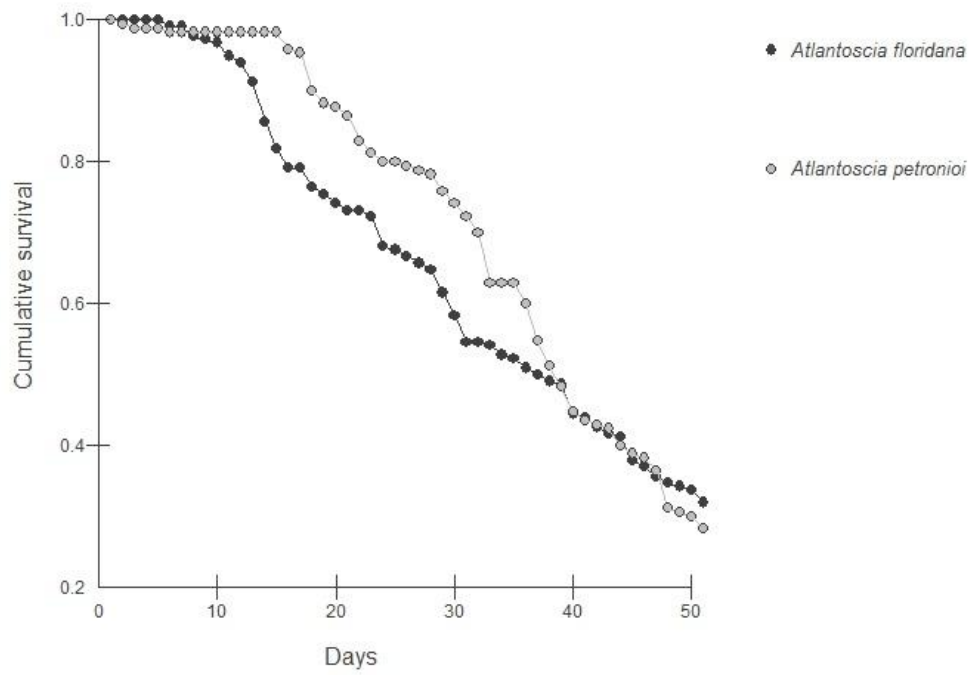
Figure 4: Survival curves of the offspring of infected females of *Atlantoscia petronioi* and non-infected females of *Atlantoscia floridana.*

Capítulo III

"*Wolbachia* in Neotropical terrestrial isopods"

Artigo submetido para a revista *FEMS Microbiology Ecology* (ISSN: 1574-6941)

Wolbachia in Neotropical terrestrial isopods

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Abstract

Wolbachia is widespread among terrestrial isopods; although, studies on this symbiotic relationship are still incipient in the Neotropical region. The aims of the present study were to investigate the presence and prevalence of _Wolbachia_ in natural populations of native and introduced species of terrestrial isopods in South America, and to analyze the diversity and phylogenetic relationships of _Wolbachia_ strains based on _16S rDNA_, _dnaA_ and multilocus sequence typing (MLST) markers. A total of 1172 individuals representing 11 families and 35 species (26 native species and nine introduced species), collected in Argentina, Brazil and Uruguay, were screened for _Wolbachia_ infection. Sixteen species were positive for _Wolbachia_; in eight of them this is the first report of the infection. Despite the general low prevalence of infection, the strains found in native species of terrestrial isopods showed high diversity and none of them belonged to the _Oni_clade, although most belonged to supergroup B. The presence of _Wolbachia_ strains from supergroups A and F was also detected for the first time in terrestrial isopods, revealing a tremendous _Wolbachia_ diversity, never before observed for this group.

Key-words: Oniscidea, South America, MLST, infection prevalence, supergroups diversity.

1. Introduction

Wolbachia (Alphaproteobacteria, Rickettsiales) is a diverse group of obligatory intracellular and maternally transmitted bacteria (Saridaki, 2010). These bacteria are known for infecting a variety of arthropods and filarial nematodes and for manipulating the reproduction of their hosts in order to ensure their own transmission to the next generations (Werren *et al.*, 2008). The frequency of infection in terrestrial arthropods was recently estimated at nearly 40% (Zug & Hammerstein, 2012), which would make *Wolbachia* the most abundant endosymbiont on Earth. It reached the status of a worldwide pandemic because *Wolbachia* strains are capable of being horizontally transmitted quite regularly. More specifically, this conclusion is due to the fact that phylogenies of *Wolbachia* strains and their arthropod hosts are incongruent (Raychoudhury *et al.*, 2009). However, in most *Wolbachia* - host systems, routes of transmission that model the endosymbiont distribution patterns remain unclear (Gerth *et al.,* 2013).

A great variety of *Wolbachia* strains is known and, according to their phylogenetic similarity, they have been assembled into supergroups, with new supergroups being continuously discovered (Augustinos *et al.*, 2011). Supergroups A and B were the first to be described and include most strains that infect arthropods (Werren *at al.*, 1995), while supergroups C and D include strains that infect only nematodes (Bandi *et al.*, 1998). Supergroup F, in turn, is the only one that comprises strains that infect both arthropods and nematodes (Casiraghi *et al.*, 2005). In order to make it easier to differentiate among *Wolbachia* strains, a multilocus sequence typing system (MLST) was developed to characterize these strains unequivocally (Baldo *et al.*, 2006a), which has helped to elucidate the paths and processes through which such diversity has been evolving (Ros *et al.*, 2009). *Wolbachia* is widespread among terrestrial isopods (Crustacea, Oniscidea).

The first molecular identification of this bacterium was in 1992, in populations of *Armadillidium vulgare* and *Porcellio dilatatus* from France (Rousset *et al.*, 1992). Since then, *Wolbachia* infection has been found in some tens of species, especially in Europe (Bouchon *et al.*, 1998; Nyirõ *et al.*, 2002; Cordaux *et al.*, 2012), but also in Africa (Ben Afia Hatira *et al,*. 2008; Cordaux *et al.*, 2012) and Asia (Wiwatanaratanabutr *et al.*, 2009). According to estimates, the prevalence of

Wolbachia infection in terrestrial isopods is nearly 61% (Bouchon *et al*., 2008). All *Wolbachia* strains known so far in terrestrial isopods belong to supergroup B, based on *16S rDNA*, *ftsZ*, *groE* and *wsp* gene analysis (Bouchon *et al.,* 1998; Cordaux *et al.,* 2001; Wiwatanaratanabutr *et al.,* 2009; Cordaux *et al.,* 2012). Additionally, most strains have great phylogenetic similarity and belong to a group known as *Oni*clade, which suggests an ancestral *Wolbachia* acquisition in this group (Cordaux *et al*., 2004; Cordaux *et al*., 2012).

In the neotropical region, more specifically in South America, studies on the symbiotic relationship between *Wolbachia* and terrestrial isopods are still incipient (Almerão *et al*., 2012; Zimmermann *et al.,* 2012), especially considering the (underestimated) diversity of more than 120 species described (Magrini *et al.,* 2010). The aims of the present study were: (i) to investigate the presence and prevalence of *Wolbachia* in natural populations of native and introduced species of terrestrial isopods in South America; (ii) to analyze the diversity of *Wolbachia* strains infecting these same species, based on the use of gene markers *16S rDNA*, *dnaA* and MLST; and (iii) to examine the phylogenetic relationships between *Wolbachia* strains found in the study.

2. Material and methods

2.1 Sample collection and DNA extraction

The species of terrestrial isopods examined in this study and the sampling sites are listed in Table 1. Natural populations of terrestrial isopods were collected during 2009 to 2012 in Brazil, Argentina and Uruguay (Figure 1). The terrestrial isopods were identified based on morphological criteria and stored in 100% ethanol at -20 °C. Dissections were performed as previously described by Bouchon *et al.* (1998) and total DNA was extracted with Chelex protocol (Bio-Rad) and PureLink Genomic DNA Kit (Invitrogen/K1820-01) for small specimens.

2.2 *Wolbachia* amplification and sequencing

Wolbachia detection was based on the amplification of the *16S rDNA* gene with the specific primers *99F* and *994R* (O'Neill *et al*., 1992). This gene is widely used for detection of *Wolbachia* and is

one of the most efficient for this purpose (Marcon *et al.*, 2011; Simões *et al.*, 2011; Almerão *et al.*, 2012). DNA quality was verified by amplification with the universal primers of the mitochondrial gene Cytochrome Oxidase I (*COI*) (Folmer *et al.*, 1994). All the reactions were carried out in volumes of 25 µl, using 1.0 µl of DNA, 0.16 µl of Taq Platinum (5U/µl), 2.5 µl of 10X buffer, 1.66 µl of MgCl$_2$ (50 mM), 0.5 µl of each primer (20 µM), 0.5 µl of dNTPs (10 mM) and 18.18 µl of ultrapure water. For the *COI* amplifications, the following settings were used: 35 cycles (50 s to 95 °C, 50 s to 57 °C and 50 s to 72 °C) with initial denaturation at 95 °C for 2 min and final extension at 72 °C for 5min. For the *16S rDNA* gene the settings were as follows: 35 cycles (1 min at 95 °C, 1 min at 50.6 °C and 1 min at 72 °C) with initial denaturation at 95 °C for 2 min and final extension at 72 °C for 5 min. PCR reactions were electrophoresed on a 1.0% agarose gel. *Wolbachia* from samples positive for the *16S rDNA* gene were subsequently identified according to *dnaA* gene (primers *dnaA2F* and *dnaA2R*, as previously described by Baldo *et al.*, 2006b) and the MLST scheme (*gatB*, *coxA*, *hcpA*, *fbpA* and *ftsZ*, as previously described by Baldo *et al.,* 2006a). Sequences were obtained using BigDye technology by Macrogen Inc., South Korea.

2.3 Phylogenetic analysis

The *dnaA*, *16S rDNA* and concatenated MLST sequences obtained in this study were aligned with sequences available in GenBank (http://www.ncbi.nlm.nih.gov) and *Wolbachia* MLST database (http://pubmlst.org/wolbachia) using ClustalW, (Thompson *et al.*, 1994) implemented in MEGA version 6.0 (Tamura *et al.,* 2013). The final alignments consisted of 343 bp for *dnaA*, 702 bp for *16S rDNA* and 2,079 bp for concatenated MLST gene sequences. Only the strains with full STs (complete five MLST alleles) were selected to construct the phylogenetic tree for the concatenated data set. The strains with incomplete allelic profiles were therefore omitted from the concatenated analysis. jModeltest version 2.1.3 (Darriba *et al.,* 2012) was used to select the model of nucleotide substitution via the Akaike Information Criterion (AIC) (Akaike, 1974). For *dnaA* and *16S rDNA* genes the GTR+I+G model were selected. For concatenated MLST data set the model selected was TPM+G. Unrooted phylogenetic trees were constructed using Maximum Likelihood (ML) method for the two

genes (*dnaA* and *16S rDNA*) and concatenated data set of MLST genes. ML trees were constructed using MEGA 6.0 (Tamura *et al.*, 2013) with 1,000 bootstrap replicates.

2.4 Genetic divergence and recombination analysis

The genetic divergence of *Wolbachia* strains (complete MLST allelic profiles) of native and introduced terrestrial isopods and pairwise genetic distance of host mitochondrial *COI*, were calculated with Kimura two parameter (K2P) model in MEGA 6.0 (Tamura *et al.*, 2013). The correlation between genetic divergence of *Wolbachia* strains and host mitochondrial genetic distance was tested by a Mantel test in the program Bioestat version 5.0 (Ayres *et al.*, 2007). Recombination analyses were conducted on single *16S rDNA*, *dnaA* and MLST loci alignments using the RDP3 package (Martin *et al.,* 2010). The default settings were used, sequences were considered linear and the highest acceptable P value cutoff was 0.05. All recombination events detected by the program were visually inspected and only recombination events detected by more than one method were listed.

2.5 Nucleotide sequence accession numbers. All *dnaA*, *16S rDNA* and *COI* gene sequences generated in this study were deposited into GenBank under accession numbers KJ814199 to KJ814239.

3. Results

3.1 *Wolbachia* infection in terrestrial isopods

A total of 1172 individuals representing 35 species (26 native species and nine introduced species) belonging to 11 families of terrestrial isopods were screened for *Wolbachia* by polymerase chain reaction (PCR) assay using *Wolbachia*-specific *16S rDNA* gene primers. The infection status of each species and the number of individuals screened are listed in Table 1. Sixteen species representing nine families were positive for *Wolbachia*. In total, 81 of the 1172 individuals were positive, 7.9% of females and 4% of males (Table 1). This was the first report of *Wolbachia* in eight of the species positive for the infection: *Atlantoscia floridana*, *Atlantoscia* sp., *Benthana taeniata*,

Pudeoniscus obscurus, *Neotroponiscus littoralis*, *Novamundoniscus gracilis*, *Trichorhina argentina,* and *T. tomentosa*. Moreover, no multiple *Wolbachia* infections were observed, i.e., only a single strain was detected in individual terrestrial isopods.

3.2 Genotyping terrestrial isopod *Wolbachia* strains

16S rDNA Wolbachia gene of all positive samples was sequenced (except for that of *Novamundoniscus gracilis*, which showed a positive band on agarose gel electrophoresis but did not result in a good sequence). These same samples were also amplified with primers of *dnaA* and MLST genes (*ftsZ*, *coxA*, *fbpA*, *hcpA* and *gatB*). Despite repeated efforts, it was not possible to amplify all the samples using all the primers tested, and primers achieved varied efficiency according to the species examined. As for the *dnaA* gene, it was not possible to obtain sequences for five of the species positive for *Wolbachia* infection: *Benthana taeniata*, *Novamundoniscus gracilis, Pudeoniscus obscurus*, *Trichorhina tomentosa*, and *T. argentina.*

According to the current approach for *Wolbachia* strain genotyping, it was possible to obtain allelic profiles or sequence types (ST) for nine of the *Wolbachia*-positive species (ten allelic profiles in total, nine of which were new, based on the available data in the *Wolbachia* MLST database). Sequence analysis revealed the presence of eleven alleles for *hcpA*, ten alleles for *gatB*, nine alleles for *ftsZ* and *fbpA*, and seven alleles for *coxA*. Sequence analysis also indicated the presence of novel alleles: nine for *gatB* and *hcpA*, seven for *ftsZ*, and six for *coxA* and *fbpA* (Table 2).

3.3 Phylogenetic analysis

Phylogenetic analysis based on *16S rDNA* (Figure 2) and *dnaA* (Figure 3) genes and on the concatenated dataset for all MLST loci (Figure 4) revealed that most *Wolbachia* strains found in terrestrial isopods collected in South America belong to supergroup B, except strains found in the introduced species *Burmoniscus meeusei* and in the native species *Neotroponiscus littoralis*, which belong to supergroups A and F, respectively. It was the first time that *Wolbachia* strains not belonging to supergroup B were observed in terrestrial isopods. All phylogenetic reconstructions

showed similar topologies, although the phylogeny generated from the *16S rDNA* sequences had low support for supergroups A and B. The strains found in introduced species of terrestrial isopods (except for that of *B. meeusei*) were very closely related and were grouped together with strains of European species into the *Oni*clade. Conversely, none of the strains found in native species of terrestrial isopods belonged to the *Oni*clade.

3.4 Genetic divergence and recombination

Mean genetic divergence of *Wolbachia* strains found in species of terrestrial isopods native to South America (all strains from supergroup B, except for one of the supergroup F) was estimated at 0.082 ±0.004, and at 0.051 ±0.003 when considering strains of the supergroup B only. With regard to the strains found in introduced species (all strains from supergroup B, except for one of the supergroup A), genetic divergence was 0.061 ±0.003, and 0.015 ±0.002 when considering strains of the supergroup B only. Table 3 shows values for genetic divergences of *Wolbachia* strains and pairwise distances of host mitochondrial *COI*. The result of the Mantel test indicated that there was a significant correlation between mitochondrial *COI* of terrestrial isopods and associated *Wolbachia* strains (r = 0.42, P = 0.01).

No recombination event was detected in the alignment of *16S rDNA* and *dnaA* genes. Conversely, two recombination events were detected in the alignment of MLST loci, one in the *gatB* gene and another one in the *fbpA* gene. In the first case, the recombinant strain was that of *Circoniscus bezzii* 1 (SiScan P <7.5×10^{-5}, RDP P <3.5×10^{-04}, BootScan P <3.6×10^{-4}, 3seq P <5.6×10^{-4}, Chimaera P <5.8×10^{-3}, GENECONV P <7.4×10^{-3}, and MaxChi P <4.9×10^{-2}). Its major and minor parents were *Burmoniscus meeusei* (94.4%) and *Balloniscus sellowii* (100%), respectively. The beginning breakpoint was position 1 in the alignment, and ending breakpoint was position 172 in the alignment. In the second case, the recombinant strain was also that of *Circoniscus bezzii* 1 (MaxChi P <7.2×10^{-7}, SiScan P <7.6×10^{-7}, 3seq P <2.6×10^{-6}, Chimaera P <2.3×10^{-5}, BootScan P <3.1×10^{-3}, and GENECONV P <9.7×10^{-3}). Its minor parent was *Burmoniscus meeusei* (98.9%) and its major parent was

unknown. The beginning breakpoint was undetermined (position 1 in the alignment), and ending breakpoint was position 189 in the alignment.

4. Discussion

4.1 Extending our knowledge on *Wolbachia* infection in terrestrial isopods

The presence of *Wolbachia* was investigated in 1172 individuals belonging to 35 species and 11 families of terrestrial isopods from South America. A screening of this magnitude has never been conducted before in natural populations of terrestrial isopods from the neotropical region. Sixteen species were infected with *Wolbachia*, twelve of them native and four introduced, and this was the first report of infection in eight of the native species.

So far, *Wolbachia* infection outside the neotropical region was known in 37 species of terrestrial isopods (Rousset *et al.*, 1992; Juchault *et al.,* 1994; Bouchon *et al.*, 1998; Nyirõ *et al.*, 2002; Ben Afia Hatira *et al*, 2008; Wiwatanaratanabutr *et al.*, 2009; Cordaux *et al.,* 2012). More recently, the presence of these bacteria was reported in five species in Brazil (Almerão *et al.*, 2012; Zimmermann *et al.*, 2012). With the inclusion of the eight new species reported in the present study, the total number of infected species of terrestrial isopods increases to 50. In addition, unlike Valette *et al.* (2013), who detected the presence of multiple *Wolbachia* infections in natural populations of *Armadillidium vulgare*, this study found only simple infections.

As for the prevalence rates of infection in the present study, although ~46% of the species were infected, it was possible to detect the presence of *Wolbachia* in only 6.9% of individuals analyzed, mostly females. In 11 of the 16 species positive for infection, less than 15% of the individuals tested had the infection. Prevalence rates found by other authors are varied, but they are usually higher than those obtained in the present study (Bouchon *et al.*, 1998; Nyirõ *et al.*, 2002; Ben Afia Hatira *et al*., 2008; Wiwatanaratanabutr *et al*., 2009). The low prevalence of *Wolbachia* infection in species of terrestrial isopods from the neotropical region may result from many factors, such as: high temperatures in the tropical and subtropical regions where animals were collected, high physiological costs associated with the lack of coadaptation between *Wolbachia* and host,

competition with other symbionts (Clancy & Hoffmann, 1998; Fleury *et al.,* 2000; Duron *et al.,* 2008).

Moreover, in the case of individuals/species considered uninfected, either *Wolbachia* density may

not be high enough to be detected using the classical PCR method (e.g., cryptic infection, Arthofer *et al*., 2009; Wolfgang *et al*., 2009) or these animals were simply not infected. Some hosts are

seemingly impervious to *Wolbachia* infection. The reasons for refractoriness are unknown, but could

be related to either bacterial or host factors (Hughes & Rasgon, 2014).

4.2 Genetic divergence and recombination analysis

Analyzes of genetic diversity corroborate phylogenetic analyses, because, when considering

Wolbachia strains from supergroup B present in introduced and native species of terrestrial isopods,

it is clear that the latter are much more diverse. The occurrence of a significant correlation between

Wolbachia divergence and host *COI* genetic distances in this study suggested the potential

cospeciation of *Wolbachia* with its terrestrial isopod hosts. A similar result was observed for other

animal groups (Raychoudhury *et al*., 2009; Yun *et al*., 2011). However, this result should be analyzed

with caution, because the number of strains used in the present study was small, and the presence of

strains from supergroups A and F in terrestrial isopods suggest the occurrence of *Wolbachia*

horizontal transfer. Thus, it seems that both codivergence and horizontal transmission contributed to

the acquisition of *Wolbachia* in terrestrial isopods.

Furthermore, recombination events appear to have had an important role in the diversity

of strains found in native species of terrestrial isopods. More specifically, the present study provides

evidence that one of the strains present in *Circoniscus bezzii* would be a recombinant of *Wolbachia*

strains from supergroups A and B. Baldo *et al.* (2006a) had already observed that *gatB* and *fbpA*

alleles had recombinants between supergroups A and B. According to the authors, these results

indicated that the two supergroups exchanged DNA frequently, although most genes showed

consistent association with one of the two supergroups. Recombination in *Wolbachia* is by no means

as rare as previously thought (Yang *et al*., 2013), and there have been records of this mechanism in a

variety of hosts groups (Werren & Bartos, 2001; Reuter & Keller, 2003; Malloch & Fenton, 2005;

Verne *et al.*, 2007; Arthofer *et al.*, 2009; Yang *et al.*, 2013). Recombination plays an important role in the evolution of bacteria in general and *Wolbachia* in particular, in ways similar to sexual reproduction of the majority of higher animals and plants (Freeman & Herron, 2007).

4.3 Phylogenetic analysis

The present study was the first to use the MLST technique to genotype *Wolbachia* strains found in terrestrial isopods of neotropical region. Although this technique can classify strains unequivocally, its efficiency was not unanimous. Augustinos *et al.* (2011) also had difficulty in amplifying MLST alleles in species of aphids. Although all the generated phylogenies were similar, that of the *16S rDNA* gene had the lowest support. According to Ros *et al.* (2009), the phylogeny of the *16S rDNA* gene is less well resolved than the phylogenies of the protein-coding genes. The authors observed evident cases of recombination and lack of support in supergroup A. These observations challenge the common assumption that the *16S rDNA* gene is a reliable phylogenetic marker that is recalcitrant to recombination.

We observed that most strains found both in native and introduced species of terrestrial isopods belong to supergroup B. However, this study also evidenced something new for terrestrial isopods, which was the presence of strains from supergroups A and F. As previously mentioned, most studies on the topic were conducted in temperate regions. In these regions, a variety of species has already been examined for the presence of *Wolbachia*, and the great majority of the strains found have such a high phylogenetic similarity (all of them belong to supergroup B) that they form a well-supported group known as *Oni*clade (Bouchon *et al.*, 2008; Cordaux *et al.*, 2012).

There are two explanations for this fact: (1) similar ecologies of related hosts facilitate transfer of related microbes, e.g., related *Wolbachia* spread between related hosts by means of shared diets or parasites (Kittayapong *et al.*, 2003; Sintupachee *et al,.* 2006; Jaenike *et al.,* 2007), predator-prey interactions (Le Clec'h *et al.*, 2013) and blood contact (Rigaud & Juchault, 1995); and/or (2) *Wolbachia* are genetically specialized on related invertebrates. In this case, *Wolbachia* are preadapted to infect related arthropods because they share similar physiologies to their current

hosts (Rigaud *et al.,* 2001; Russel *et al.,* 2009). Thus, strains present in palearctic species of terrestrial isopods seems to be specialized and well established in host populations. Indeed, the only strains belonging to the *Oni*clade found in this study were those present in *Armadillidium vulgare*, *Porcellio dilatatus*, and *P. laevis*. These species originate from Europe and from the Mediterranean region and were introduced in several regions of the world, especially due to human activities (Schmalfuss, 2003). In other words, even after being taken to other regions of world, these species preserved the strains acquired in their places of origin, which further reinforces the idea of strains specialized in host species.

Conversely, the scenario is quite different in South America. Cordaux *et al.* (2012) had already predicted that screenings of terrestrial isopods in regions that have not been extensively studied so far may have the potential to uncover a tremendous and unexpected *Wolbachia* diversity. None of the strains from supergroup B infecting native species belongs to the *Oni*clade; additionally, most of them are not phylogenetically related. A similar result had already been observed by Almerão *et al*. (2012) in natural populations of *Balloniscus glaber* and *B. sellowii.* Even if codivergence may have had a certain role, the great diversity of strains found in terrestrial isopods of the neotropical region would be more consistent with the occurrence of several events of loss and acquisition of *Wolbachia*. This hypothesis is reinforced by the low prevalence of infection in most species analyzed (i.e., lack of *Wolbachia*-host specialization). Beyond that, some authors have observed variable frequencies of low-density *Wolbachia* infections associated with a high diversity of strains (Sun *et al.,* 2007; Hughes *et al*., 2011), possibly as a result of the interaction with other microbial flora within the animal. These results add to an emerging understanding that *Wolbachia* may be more pervasive than currently accepted, due to cryptic infections that occur in few individuals within a population and at low infection densities within these hosts (Hughes *et al*., 2011).

The differences observed between *Wolbachia* strains of terrestrial isopods from palearctic and neotropical regions suggest that geographical origin would have a significant impact on the divergence of *Wolbachia* strains. Studies with fig wasps (Haine & Cook, 2005) and ants (Russell *et al*., 2009) have already demonstrated a considerable association between biogeography and similarity of

Wolbachia strains. The latter study found a strong split between strains from New World and non-New World ants, suggesting that geographical barriers (in this case, oceans) promote the divergence among *Wolbachia* populations, limiting gene flow and horizontal transfer among host species. A similar case may have occurred for terrestrial isopods from palearctic and neotropical regions.

The presence of distantly related *Wolbachia* strains in terrestrial isopod species, more specifically from supergroups A and F, strongly support the hypothesis that horizontal transmission of *Wolbachia* between arthropod species from unrelated taxa has occurred. The same has been already reported in the spider genus *Agelenopsis* (Baldo *et al*., 2008), in the wasp genus *Nasonia* (Raychoudhury *et al.,* 2009), in the acari genus *Bryobia* (Ros *et al*., 2009), in the termites genus *Odontotermes* (Salunke *et al.,* 2010) and in tsetse flies genus *Glossina* (Doudoumis *et al.,* 2012). *Burmoniscus meeusei* is a species introduced from Asia and found in England, Hawaii, and Brazil (Schmalfuss, 2003). Studies on the biology and ecology of this species are scarce; thus, it is very difficult to suggest the source of transmission of *Wolbachia* from supergroup A found in these individuals.

On the other hand, the presence of one strain from supergroup F in *Neotroponiscus littoralis* may be explained by the biology of these animals. Strains from supergroup F are found in nematodes and arthropods (Panaram & Marshall, 2006; Baldo *et al*., 2007; Roy & Harry, 2007; Ferri *et al*., 2011; Hughes *et al*., 2011; Morse *et al*., 2012); however, termites are the most representative host for this supergroup. Strains from the supergroup F are known in 16 species distributed worldwide, belonging to Termitidae, Rhinotermitidae, and Kalotermitidae families (Lo & Evans, 2007; Roy & Harry, 2007, Salunke *et al*., 2010). Representatives of the genus *Neotroponiscus* are small-sized animals (Lemos de Castro, 1970) that*,* contrary to most species of terrestrial isopods that prefer leaf litter, are found in native and secondary vegetation associated with tree barks (Quadros *et al*., 2007). A recent study observed that *N. carolii* specimens occupy abandoned arboreal nests of *Nasutitermes* spp. termites (Lisboa *et al*., 2013). Furthermore, the concomitant presence of *Nasutitermes* sp. termites and isopods genus *Neotroponiscus* in tree barks has already been observed in field works (BLZ, personal observation). Since there is an intimate contact between the two groups, it is plausible

to believe that termites have transmitted the strain from supergroup F to the terrestrial isopod *N. littoralis*. However, *in loco* studies are needed to confirm this hypothesis, especially considering that termites from the Brazilian native fauna have never been tested for the presence of *Wolbachia*. It is worth remembering that strains from supergroup F are also found in nematodes, but nematodes have never been reported in either *Neotroponiscus* spp. nor were found in the individuals used in this study.

5. Conclusion

This study presents the largest screening ever conducted in the neotropical region to investigate the presence of *Wolbachia* in terrestrial isopods, using the MLST technique to characterize the strains present in this animal group. The strains found in native species of terrestrial isopods showed high diversity and none of them belonged to the *Oni*clade, although most belonged to supergroup B. The presence of *Wolbachia* strains from supergroups A and F was also detected for the first time in terrestrial isopods, being found in *Burmoniscus meeusei* and *Neotroponiscus littoralis*, respectively. The present study corroborates the statement that screenings of terrestrial isopods in regions that have not been extensively studied so far have the potential to uncover a tremendous and unexpected *Wolbachia* diversity.

6. Acknowledgments

7. References

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Table 1: Terrestrial isopods species and collection sites. Species in bold are native to their collection sites. Asterisks indicate species in which we detected the presence of *Wolbachia* infection. FT: number of females tested, FI: number of females infected, MT: number of males tested, MI: number of males infected. BR: Brazil; AR: Argentina; UY: Uruguay. Other sets of letters correspond to Brazilian states (RS: Rio Grande do Sul; SC: Santa Catarina; PR: Paraná; SP: São Paulo; MG: Minas Gerais; DF: Distrito Federal; BA: Bahia; PB: Paraíba; CE: Ceará; MA: Maranhão; PA: Pará).

Family	Species	Collection Site (Municipality)	FT	FI	MT	MI	Prevalence
Armadillidae	*Cubaris murina*	Foz do Iguaçu (PR/BR)	23	0	12	0	0%
	Cubaris sp.	Chapadinha (MA/BR)	2	0	3	0	0%
Armadillidiidae	*Armadillidium nasatum*	Porto Alegre, Barra do Ribeiro, Santa Rosa (RS/BR); Montevidéu, Punta Del Leste (UY)	22	0	26	0	0%
	Armadillidium vulgare*	Barra do Ribeiro, Candiota, Porto Alegre, Santa Rosa (RS/BR); Boqueirão (PB/BR); Varginha (MG/BR); Montevidéu, Punta Del Leste (UY); Buenos Aires (AR)	80	19	72	0	12.5%
Balloniscidae	**Balloniscus glaber***	Mariana Pimentel, Mostardas, Porto Alegre (RS/BR)	33	2	11	1	6.8%
	Balloniscus sellowii*	Arroio do Sal, Barra do Ribeiro, Mostardas, Pelotas, Porto Alegre, Vacaria (RS/BR); Bocaína do Sul (SC/BR); Lapa (PR/BR); Montevidéu (UY)	84	4	27	4	7.2%
Bathytropidae	**Neotroponiscus littoralis***	Ilhéus (BA/BR)	5	3	1	0	50%
	Neotroponiscus sp.	Mariana Pimentel (RS/BR)	8	0	2	0	0%
Dubioniscidae	**Calycuoniscus bodkini**	Iguatu (CE/BR)	4	0	0	0	0%
	Calycuoniscus sp.	Iguatu (CE/BR)	7	0	0	0	0%
	Phalloniscus sp.	Capão do Cipó (RS/BR)	8	0	0	0	0%
	Novamundoniscus gracilis*	Porto Alegre (RS/BR)	10	1	4	0	7.1%
Philosciidae	**Alboscia** sp.	Antonina (PR/BR)	7	0	6	0	0%
	Atlantoscia floridana*	Arroio do Sal, Barra do Ribeiro, Porto Alegre, Mariana Pimentel, Mostardas, Torres (RS/BR); Blumenau, Corupá, Orleans, Rio Fortuna, São João Batista (SC/BR); Curitiba, Foz do Iguaçu (PR/BR); Varginha (MG/BR)	198	2	34	2	1.7%
	Atlantoscia petronioi*	Porto Alegre (RS/BR)	22	19	8	4	76.7%
	Atlantoscia rubromarginata	Ilhéus (BA/BR)	15	0	5	0	0%
	Atlantoscia sp. 1*	Orleans (SC/BR)	27	4	2	0	13.8%
	Atlantoscia sp. 2	Blumenau, São João Batista (SC/BR)	26	0	5	0	0%
	Benthana cairensis	Corupá (SC/BR); Quitandinha (PR/BR); Taquara (RS/BR)	20	0	8	0	0%
	Benthana itaipuensis	Foz do Iguaçu (PR/BR)	2	0	0	0	0%
	Benthana picta	Curitiba (PR/BR); Ilhéus (BA/BR); Pelotas (RS/BR)	12	0	0	0	0%
	Benthana taeniata*	Augusto Pestana, Capão do Cipó (RS/BR); Antonina, Piên (PR/BR); Conceição de Ibitipoca (MG/BR); São Carlos (SP/BR); Santa Rosa de Lima (SC/BR)	110	1	17	0	0.8%
	Benthana sp. 1	Barra do Ribeiro (RS/BR)	5	0	1	0	0%
	Benthana sp. 2	Brasília (DF/BR)	6	0	2	0	0%
	Burmoniscus meeusei*	Porto Alegre (RS/BR)	2	2	0	0	100%
Platyarthridae	**Trichorhina acuta**	Rio Fortuna (SC/BR)	3	0	0	0	0%
	Trichorhina argentina*	Porto Alegre (RS/BR)	23	4	6	0	13.8%
	Trichorhina tomentosa*	Porto Alegre (RS/BR); Varginha (MG/BR)	18	1	0	0	5.5%
Porcellionidae	*Porcellio dilatatus**	Porto Alegre (RS/BR); Punta Del Leste (UY)	10	1	7	1	11.8%
	Porcellio laevis*	Augusto Pestana (RS/BR)	12	2	12	0	8.3%
	Porcellionides pruinosus	Augusto Pestana (RS/BR); Jaraguá do Sul, Rio Fortuna (SC/BR)	40	0	25	0	0%
Pudeoniscidae	**Pudeoniscus obscurus***	Ilhéus (BA/BR)	2	1	0	0	50%
Scleropactidae	**Circoniscus bezzii***	Presidente Olegário (MG/BR)	7	3	0	0	42.9%
	Circoniscus gaigei	Belém (PA/BR)	1	0	2	0	0%
Styloniscidae	*Styloniscus otakensis*	São Francisco de Paula (RS/BR)	20	0	0	0	0%
		Total	**874**	**69**	**298**	**12**	

Table 2: MLST allelic profiles of *Wolbachia* detected in terrestrial isopods species from South America. Asterisk indicates alleles that were new to the MLST database.

Species	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*	ST
Armadillidium vulgare 1	13	13	14	9	13	6
Armadillidium vulgare 2	13	13	248*	9	13	411
Atlantoscia floridana	219*	207*	242*	189*	397*	412
Atlantoscia petronioi	220*	-	243*	-	-	
Atlantoscia sp.	220*	207*	244*	190*	398*	413
Balloniscus glaber	221*	213*	-	-	-	
Balloniscus sellowii 1	221*	208*	6	191*	183	415
Balloniscus sellowii 2	221*	208*	6	191*	-	
Benthana taeniata	-	-	-	-	402*	
Burmoniscus meeusei	222*	210*	249*	32	282	414
Circoniscus bezzii 1	223*	209*	245*	192*	399*	416
Circoniscus bezzii 2	224*	-	-	-	-	
Neotroponiscus littoralis	228*	214*	246*	196*	400*	417
Novamundoniscus gracilis	-	-	247*	-	-	
Porcellio dilatatus	225*	13	250*	193*	401*	418
Porcellio laevis	226*	13	14	194*	13	419

Table 3: Mitochondrial *COI* pairwise genetic distance and genetic divergence of *Wolbachia* strains (complete STs) among terrestrial isopods species. The mitochondrial *COI* pairwise distances and genetic divergence of *Wolbachia* strains are given above and below the diagonal, respectively.

|		A. vulgare	A. vulgare	A. floridana	Atlantoscia sp.	B. sellowii	B. meeusei	C. bezzii	N. littoralis	P. dilatatus	P. laevis
A. vulgare	ST 6	–	0.050	0.229	0.297	0.226	0.262	0.301	0.281	0.246	0.227
A. vulgare	ST 411	0.000	–	0.235	0.299	0.235	0.262	0.324	0.309	0.255	0.241
A. floridana	ST 412	0.070	0.071	–	0.167	0.203	0.235	0.320	0.314	0.230	0.256
Atlantoscia sp.	ST 413	0.073	0.074	0.011	–	0.269	0.285	0.370	0.339	0.311	0.298
B. sellowii	ST 415	0.053	0.053	0.045	0.041	–	0.234	0.296	0.296	0.261	0.252
B. meeusei	ST 414	0.127	0.128	0.109	0.114	0.121	–	0.308	0.284	0.274	0.249
C. bezzii	ST 416	0.087	0.088	0.078	0.075	0.060	0.107	–	0.325	0.302	0.339
N. littoralis	ST 417	0.131	0.131	0.138	0.136	0.122	0.135	0.120	–	0.326	0.280
P. dilatatus	ST 418	0.024	0.024	0.085	0.086	0.066	0.129	0.098	0.130	–	0.247
P. laevis	ST 419	0.014	0.015	0.085	0.086	0.067	0.128	0.098	0.130	0.016	–
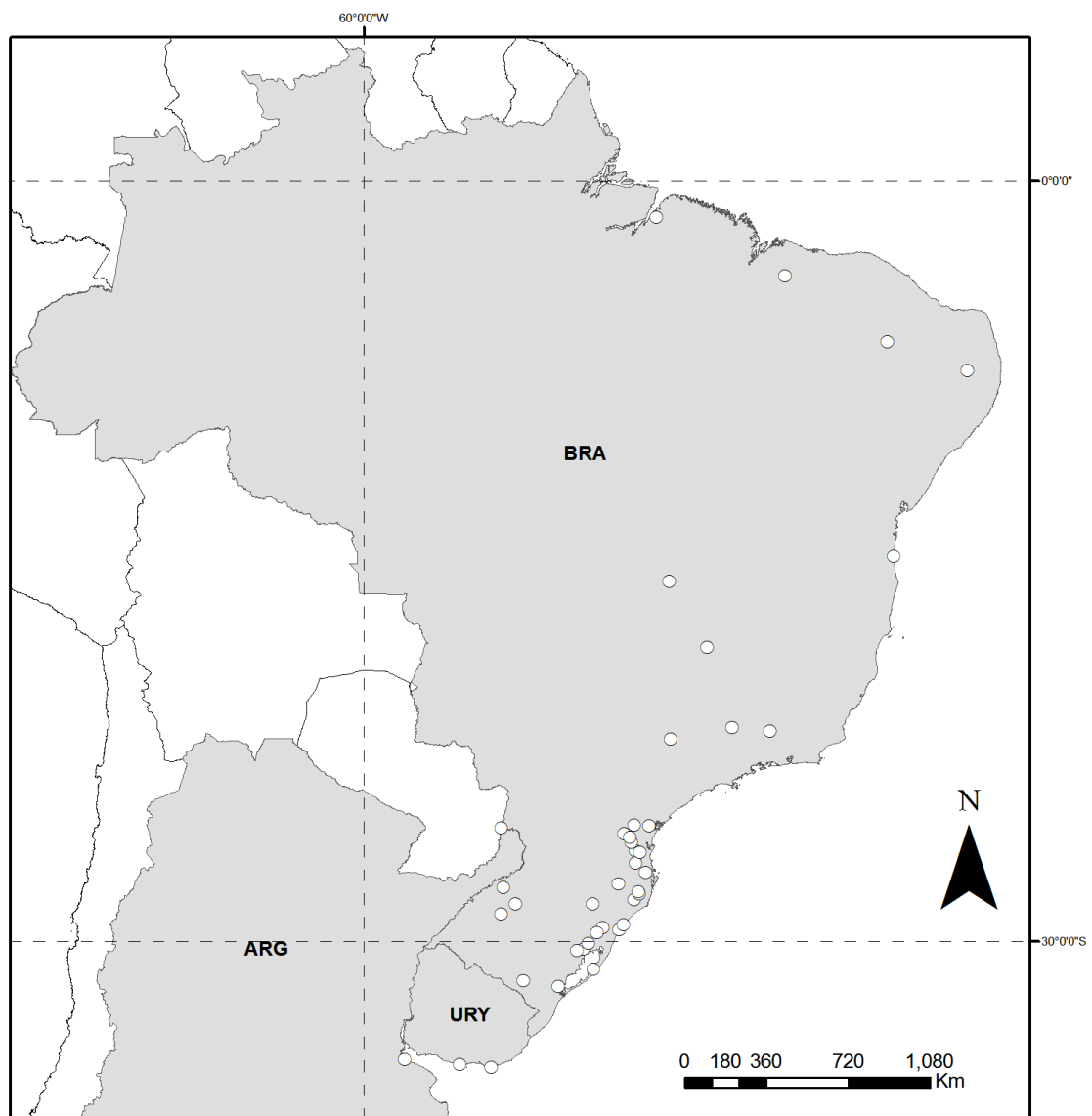
Figure 1: Collection sites (circles) of terrestrial isopod species examined for *Wolbachia* infection in South America.
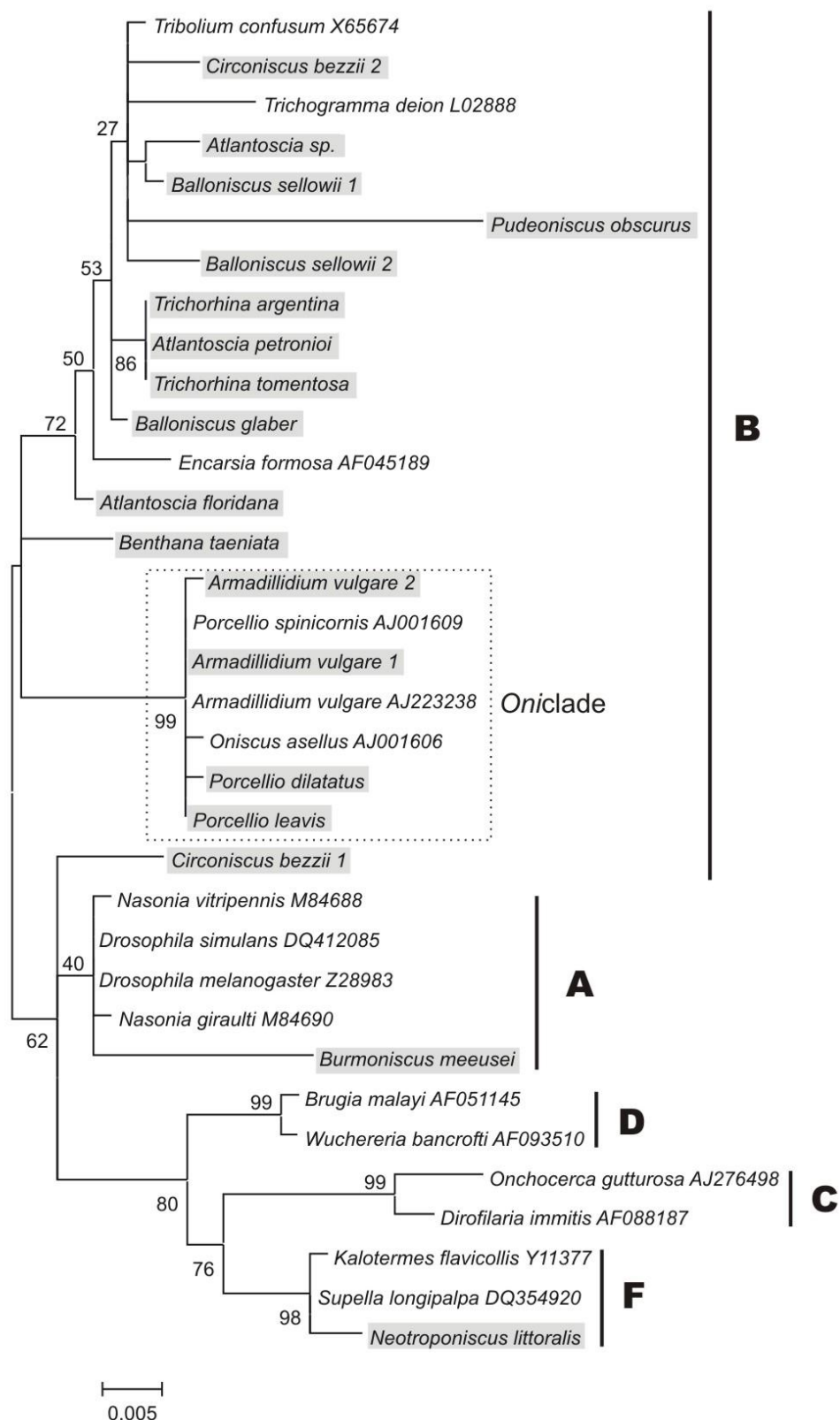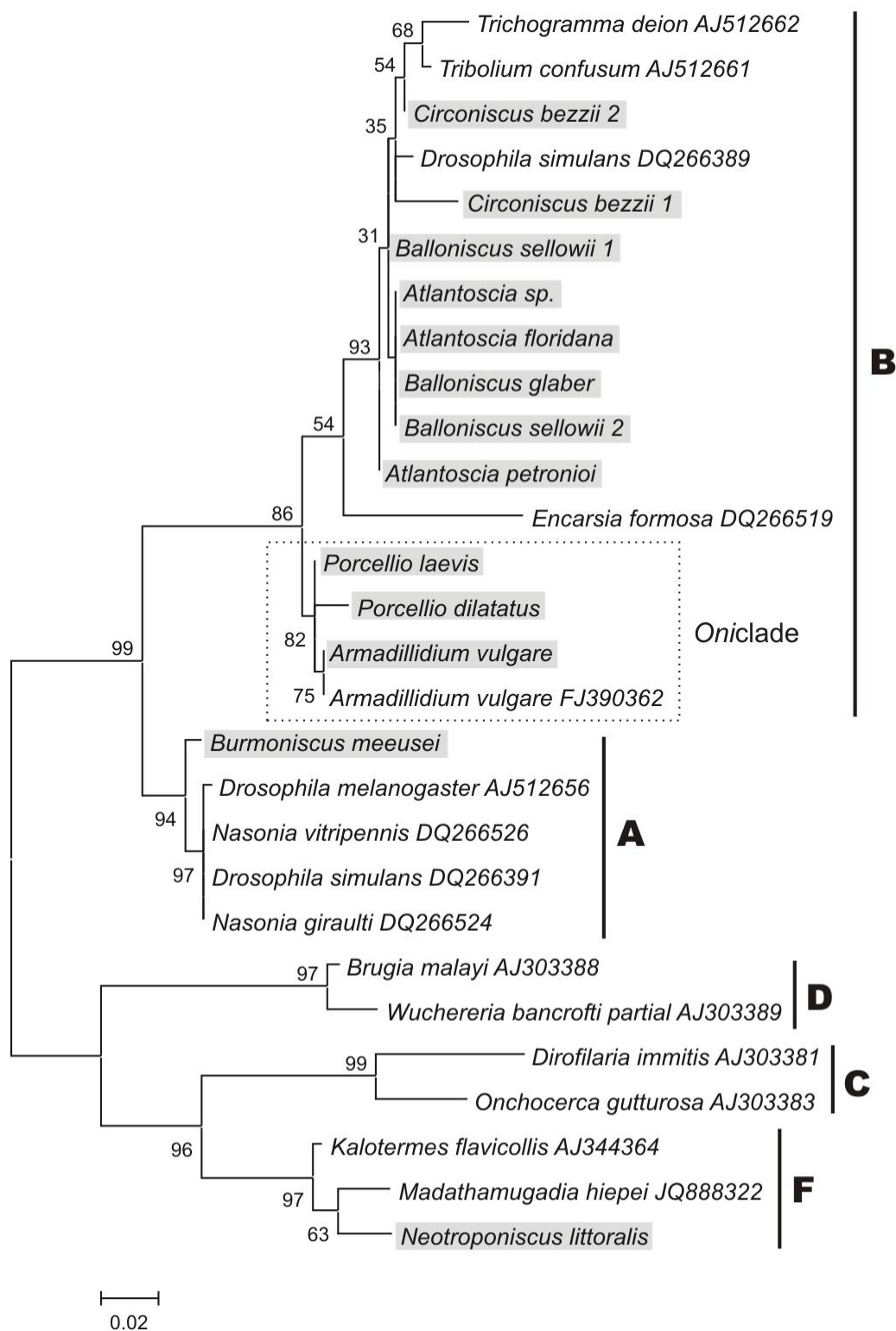
Figure 2: Unrooted maximum-likelihood phylogenetic analysis of *16S rDNA* gene sequences. Numbers at nodes indicate bootstrap support values (1000 replicates). Species highlighted in the tree correspond to *Wolbachia* strains identified in the present study. Alphanumeric codes represent GenBank ID numbers. Letters represent *Wolbachia* supergroup designations.

Figure 3: Unrooted maximum-likelihood phylogenetic analysis of *dnaA* gene sequences. Numbers at nodes indicate bootstrap support values (1000 replicates). Species highlighted in the tree correspond to *Wolbachia* strains identified in the present study. Alphanumeric codes represent GenBank ID numbers. Letters represent *Wolbachia* supergroup designations.
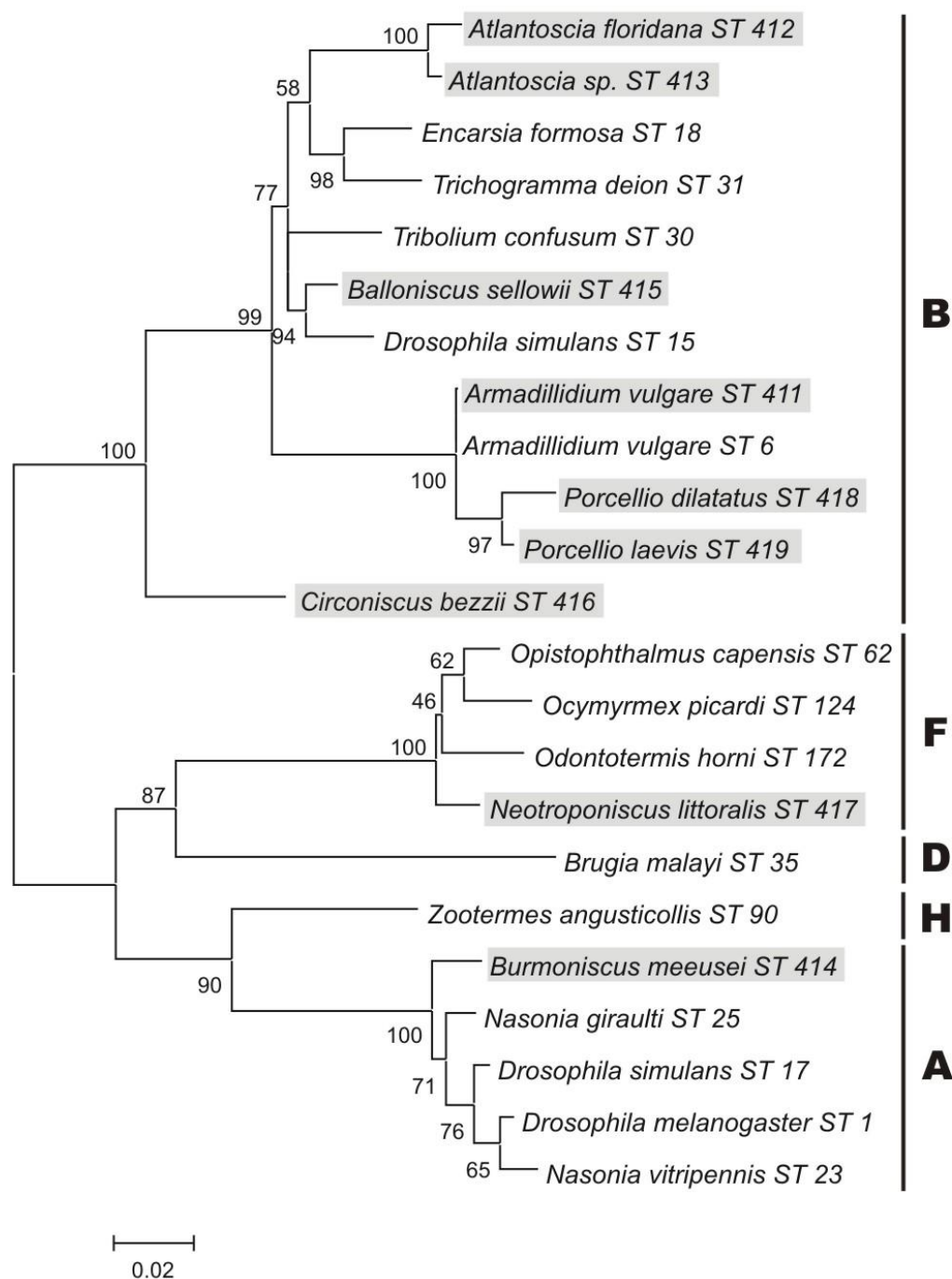
Figure 4: Unrooted maximum-likelihood phylogenetic analysis of a 2079 bp alignment of five *Wolbachia* MLST concatenated gene sequences (*coxA*, *gatB*, *hcpA*, *ftsZ* and *fbpA*). Numbers at nodes indicate bootstrap support values (1000 replicates). ST numbers represent MLST allelic profiles (searchable at http://pubmlst.org/wolbachia/). STs highlighted in the tree correspond to those identified in the present study. Letters represent *Wolbachia* supergroup designations.

Capítulo IV

"Investigating the taxonomy and molecular phylogeny of the

Neotropical genus *Atlantoscia* (Oniscidea, Philosciidae):

DNA barcoding and description of two new species"

Investigating the taxonomy and molecular phylogeny of the Neotropical genus *Atlantoscia* (Oniscidea, Philosciidae): DNA barcoding and description of two new species

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Abstract

Differences between nominal species in the genus *Atlantoscia* are generally small and restricted to a few characters. Despite the utility of DNA sequences to identify and distinguish between species, molecular phylogenies were never made for terrestrial isopods from Neotropics. The aims of this study were to describe two new species of *Atlantoscia*, and to use molecular markers in order to verify the validity of the current taxonomy and the relationships between the species within this genus. All of the *Atlantoscia* species showed a strong support in the generated phylogenetic trees. The average congeneric distance was of 14.7%, with the species *A. ituberasensis* and *A. rubromarginata* presenting the highest values of genetic divergence. Thus, the present study shows that the COI gene, used in DNA barcoding, is a useful tool, capable of distinguishing between *Atlantoscia* species with quite reliability. In this way, DNA barcoding could be helpful in those cases where the classical taxonomy does not provide a clear-cut resolution.

1. Introduction

Nowadays, the genus *Atlantoscia* Ferrara & Taiti, 1981 (Philosciidae) consists of five species, *A. floridana* (Van Name 1940) originally described from Florida, United States; *A. rubromarginata* Araujo & Leistikow, 1999 from Sergipe, Brazil (Araujo & Leistikow 1999); *A. petronioi* Campos-Filho, Contreira & Lopes-Leitzke, 2012 from Rio Grande do Sul, Brazil; and the ones described most recently *A. sulcata* and *A. ituberasensis* Campos-Filho, Lisboa & Araujo, 2013, from the Brazilian states of São Paulo and Bahia, respectively.

Atlantoscia floridana is the only species with a wide distribution. Its geographical range includes coastal regions of several Brazilian states (Amapá, Pará, Tocantins, Rio Grande do Norte, Paraíba, Pernambuco, Bahia, Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, Santa Catarina and Rio Grande do Sul), Florida (USA) and Argentina, as well as Ascension and St. Helena islands (Souza-Kury, 1998; Schmalfuss, 2003; Campos-Filho *et al.*, 2013). In terms of habitat, they are generalist animals which can occur in distinct environments, and frequently in large numbers (Lopes *et al.*, 2005; Quadros *et al.*, 2009). Furthermore, they present all the attributes of a pioneer species, such as quick growth, early breeding and a great investment in fecundity (Quadros *et al.*, 2009).

One of the most commonly characters used to identity *A. floridana* is its color pattern, more specifically the presence of a non-pigmented inverted U-shaped band on the cephalothorax. Nonetheless, some specimens are described as not showing this pattern (Lemos de Castro, 1985; Campos-Filho et al., 2013). Such characteristics, as well as the great morphological similarity between some species of the genus (e.g., *A. floridana* and *A. petronioi*) make it difficult to properly identify the animals (Zimmermann *et al.*, *submitted*).

Taxonomy on species level for terrestrial isopods has been based mainly in a few male sexual characters (Parmakelis *et al.*, 2008). Besides, taxonomic discrimination of closely related species is often based on subtle morphological features, which difficult routine identification (Brökeland & Raupach, 2008). DNA sequences, especially the COI gene used in DNA barcoding, can be used to identify and distinguish between crustacean species (Costa *et al.*, 2007). Their usage

combined with a morphological analysis may be useful to reduce the problems associated with the isolated use of the morphological techniques, which could underestimate the true levels of divergence between populations (Klossa-Kilia, *et al.*, 2005, 2006). DNA barcoding is based on the premise that a short standardized sequence can distinguish individuals of a species because genetic variation between species exceeds that within species (Hebert *et al.*, 2003a). The usability and robustness of COI in a standard high-throughput barcoding analysis have been extensively assessed (Hajibabaei *et al.*, 2005).

Phylogenetic relationships among species of terrestrial isopods are still largely unknown because robust analyses have started to appear only relatively recently (Parmakelis *et al.*, 2008). These analyses, that at first focused on the phylogeny of higher taxonomic groups (Wetzer, 2001, 2002; Mattern, 2003), now aim at the resolution of the relationships between congeneric species or between populations from a nominal species (Rivera *et al.*, 2002; Charfi-Cheikrouha, 2003; Klossa-Kilia *et al.*, 2006; Parmakelis *et al.*, 2008; Poulakakis & Sfenthourakis, 2008; Karasawa & Honda, 2012; Lee *et al.*, 2014; Kamilari *et al.,* 2014). Phylogenetic studies of terrestrial isopods have primarily focused on species from Northern Hemisphere, with a few rare exceptions (e.g. Cooper *et al.*, 2008; Lee *et al.*, 2014).

Molecular phylogenies of terrestrial isopods have never been carried out in the Neotropical region. Thus, the objective of this work was to use molecular markers, more specifically the mitochondrial COI gene, in order to verify the validity of the current taxonomy and the phylogenetic relationships between the species within the genus *Atlantoscia*. The current study also brings the description of two new species from this same genus, including the molecular analysis of both species.

2. Material and methods

2.1 Field Sampling

Terrestrial isopods were collected along five Brazilian states and sampling locations were recorded using a GPS handset. For molecular analyzes, isopods collected were preserved in 95%

ethanol. Individuals from scientific collections, preserved in 70% ethanol, were also utilized (Coleção de Carcinologia UFRGS, Table 1). For inclusion as out-groups, we collected and sequenced individuals of *Burmoniscus meeusei* (Holthuis, 1947) (Philosciidae) and *Neotroponiscus littoralis* Lemos de Castro, 1970 (Bathytropidae) (Table 1). Specimens used for morphological analysis were collected in states of Rio Grande do Sul (Porto Alegre, 30°12'30"S 51°10'10"W), Santa Catarina (Orleans, 28°21'17"S 49°16'29"W) and Paraná (Morretes, 25°26'46"S 48°46'52"W; Parque Estadual Saint-Hilaire/Lange, 25°38'39"S 48°36'04), stored in 70% ethanol and descriptions were based on morphological characters.

2.2 Morphological Analysis: description of two new species of *Atlantoscia*

The specimens were dissected and the appendages and pereonites were mounted on slides. Drawings were prepared using a camera lucida. The *noduli laterales* were measured and illustrated as in Vandel (1962). The material was deposited in the Museu de Zoologia (MZUSP), Universidade de São Paulo, São Paulo; and Coleção de Carcinologia (UFRGS), Universidade Federal do Rio Grande do Sul, Rio Grande do Sul.

2.3 DNA extraction and PCR amplification

Dissections were conducted as previously described by Bouchon *et al*. (1998). Total DNA was extracted with Chelex protocol (Bio-Rad) and PureLink Genomic DNA Kit (Invitrogen/K1820-01) for individuals preserved in ethanol 70%. We performed PCR amplifications using the set of primers LCO/HCO (Folmer *et al*., 1994), which amplify a fragment of approximately 700 bp of mitochondrial gene cytochrome c oxidase subunit I - COI. All the reactions were carried out in volumes of 25 μl, using 1.0 μl of DNA, 0.16 μl of Taq Platinum (5U/μl), 2.5 μl of 10X buffer, 1.66 μl of MgCl$_2$ (50 mM), 0.5 μl of each primer (20 μM), 0.5 μl of dNTPs (10 mM) and 18.18 μl of ultrapure water. For the amplifications, the following settings were used: 35 cycles (50 s to 95 °C, 50 s to 57 °C and 50 s to 72 °C) with initial denaturation at 95 °C for 2 min and final extension at 72 °C for 5 min. PCR reactions were electrophoresed on a 1.0% agarose gel. All DNA purification and sequencing was carried out by

Macrogen (Seoul, South Korea), using BigDye technology, with each sample being sequenced in both the forward and reverse directions using the same primers as those used in amplification.

2.4 Sequence alignment and genetic divergence analysis

The electropherograms obtained were assembled, edited and visualized in the Staden package (Staden, 1996). Subsequently, the consensus sequences were aligned using the Clustal W algorithm, implemented in MEGA version 6.0 (Tamura *et al*., 2013). The final alignment of the COI data set was verified against published COI sequences of other isopod species available in GenBank. The COI sequences produced in the present study were unambiguously aligned to the retrieved COI sequences, whereas no gaps and/or stop codons were present the mtDNA sequence data set. Therefore, the authenticity of the produced mtDNA sequences was verified.

The pairwise genetic divergences of COI sequences were calculated with Kimura two parameter (K2P) model in MEGA 6.0 (Tamura *et al*., 2013). The average values of intraspecific and interspecific distance were obtained using this same software, with 10,000 bootstrap replicates, as suggested by Hebert *et al*. (2003b) for DNA barcoding. All COI gene sequences generated in this study were deposited into GenBank database (http://www.ncbi.nlm.nih.gov/) and the accession numbers are shown in Table 1.

2.5 Phylogenetic analysis

Phylogenetic analyses were conducted using Neighbor Joining (NJ) in MEGA 6.0 (Tamura *et al*., 2013), and using Bayesian inference (BI), in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). For NJ analyses was utilized the model of nucleotide substitution suggested by Hebert *et al.* (2003b) for DNA barcoding, with 10,000 bootstrap replicates. For Bayesian phylogenetic analysis, MrModeltest 2.2.1 (Nylander, 2004) was used to select the best-fitting substitution model for the COI alignment according to the Akaike Information Criterion (AIC - Akaike, 1974). The GTR+I+G model were selected. Posterior distributions of parameters, including the tree topology and branch lengths, were estimated using Markov chain Monte Carlo (MCMC) sampling. Samples from the posterior

distribution were drawn every 1,000 generations over a total of 1,000,000 generations. The first 25% of samples were discarded as burn-in.

3. Results

3.1 Morphological Analysis: description of two new species of *Atlantoscia*

Systematics

Family Philosciidae Kinahan, 1857

Atlantoscia Ferrara & Taiti, 1981

Type species: *Atlantoscia alceui* Ferrara & Taiti, 1981

Diagnosis: See Ferrara & Taiti (1981) and Araujo & Leistikow (1999).

Atlantoscia n. sp. 1 Campos-Filho & Araujo, 2014

Figures 1 and 2

Type material. *Holotype* male, Brazil, Rio Grande do Sul, Porto Alegre, Belém Novo, II/2012, leg. B.L. Zimmermann (MZUSP 32622). *Paratypes* same data as holotype: X ♂, X ♀♀ (MZUSP 32623), 4 ♂♂ (UFRGS 5810P). Paraná: 1 ♂, 5 ♀♀, Morretes, 10.VIII.2012, leg. P.B. Araujo & B.L. Zimmermann, in buldings, near of the road (MZUSP 32624), 2 ♂♂, 11 ♀♀, Morretes, 10.VIII.2012, leg. P.B. Araujo & B.L. Zimmermann (MZUSP 32625), 3 ♂♂, 2 ♀♀, Parque Estadual Saint-Hilaire/Lange, 10.VIII.2012, leg. P.B. Araujo & B.L. Zimmermann (MZUSP 32626), 2 ♂♂, 2 ♀♀, Parque Estadual Saint-Hilaire/Lange, 10.VIII.2012, leg. P.B. Araujo & B.L. Zimmermann (UFRGS 5811P).

Diagnosis: The main diagnostic characteristic of the new species is the male pleopod 1 endopod bearing a lobe on outer margin.

Description: Maximum body length 8.5 mm, cephalothorax width 1.8 mm. Light brown color. Cephalothorax with irregular unpigmented spots. Peduncle of antenna with third article and distal half portion of the fourth article unpigmented, flagellum completely pigmented. Pereon with

unpigmented longitudinal spots paramedially, pereonites 5-7 more pigmented; pleon with large unpigmented spot T-like, pleonites 3-5 with two unpigmented spots paramedially; epimera 1-7 with unpimented borders. Telson with three unpigmented spots.

Body (Fig. 1A) convex; dorsal surface smooth and bright, bearing few long piliform scale-setae (Fig. 1B); pleon narrower than pereon with developed epimera; pereonites 1–3 with postero-lateral corners right-angled and posterior margins straight; pereonites 4–7 with postero-lateral corners gradually more acute and posterior margins gradually more arched; pleon epimera 3–5 well developed, triangular, acute, and directed backwards. Cephalothorax (Figs 1C, D) with linea supra-antennalis centrally bent-downwards; linea frontalis and lateral lobes lacking; clypeus and labrum similar in length; eyes with 16-18 ommatidia arranged in four rows. One line of *noduli laterales* per side (Fig. 1E, F) with d/c coordinates reaching a maximum on pereonite 4; b/c coordinates gradually decreasing. Telson (Fig. 1G) triangular with lateral margins straight and right-angled apex.

Antennule (Fig. 1H) triarticulate, distal article the longest, with 8 aesthetascs plus apical pair. Antenna (Fig. 1I) when extended posteriorly reaches sixth pereonite, flagellum triarticulate, first article longest, second and third subequal in length; apical organ short, one third of the length of the distal article, free sensilla inserted near the base.

Mandibles (Fig. 1J, K) with molar penicil consisting of at least seven branches, pars intermedia with dense tuft of coniform setae, left mandible with three penicils, and right mandible with two penicils. Maxillule (Fig. 1L) medial endite with two penicils (not showed), inserted transversely, distal portion rounded; outer endite with 4+6 teeth, five teeth of inner set cleft, one of them trifid and one simple. Maxilla (Fig. 1M) lateral lobe twice as wide as medial lobe with distal margin sinuous, covered with trichiform setae; medial lobe rounded, covered with trichiform setae. Maxilliped (Fig. 1N) with rectangular base bearing sparse piliform setae; endite rectangular, outer margin rounded, distal margin sinuous, medial seta overpassing distal margin and a setose ventral sulcus lacking penicil.

Pereopods (Fig. 2A, B) rather slender, piliform setae on all articles, hyaline fringe and sparse setae on merus and carpus; carpus 1 with transverse antenna-grooming brush and one seta with

double-serrate apex; ischium 7 triangular, bearing four long setae; dactylus 1–7 with inner claw reaching distal margin of outer claw, dactylar seta long and simple, ungual seta simple, not surpassing inner claw.

Uropod (Fig. 2C) with protopod grooved on outer margin bearing glandular, endopod and exopod inserted at different levels, with few long setae along inner and outer margins; exopod slightly longer than endopod, with five setae on apex; endopod with three setae on apex.

Pleopods 1-5 with respiratory area. Male: Genital papilla (Fig. 2D) with triangular frontal shield distally acute and two subapical orifices. Pleopod 1 (Fig. 2E) exopod heart-shaped, outer margin without setae, medially concave and rounded apex; endopod slightly stout, outer distal margin bearing a lobe and inner margin with crenulate plaque. Pleopod 2 (Fig. 2F) exopod triangular, outer margin concave with about six setae; endopod slender and acute apex. Pleopods 3 and 4 exopod as in Fig. 2 G and H. Pleopod 5 exopod (Fig. 2I) triangular, outer margin slightly sinuous and bearing three setae.

Remarks: The new species can resembles *Atlantoscia floridana* by the shape of the male pleopod 1 endopod, but easily distinguished through the same structure. *Atlantoscia floridana* presents on the endopod a protrusion-like shape (see Araujo & Leistikow, 1999) differently from *Atlantoscia* n. sp. 1 which shows a lobe. Also can be distinguished from all other congeneric species by the shape of the male pleopod 1 exopod.

Atlantoscia n. sp. 2 Campos-Filho & Araujo, 2014

Figures 3 and 4

Type material: Holotype male, Brazil, Santa Catarina, Orleans, 24/VI/2012, leg. P.B. Araujo & B.L. Zimmermann (MZUSP 32627). Paratypes same data as holotype: 5 ♂♂, 5 ♀♀ (MZUSP 32628); 8 ♂♂, 25 ♀♀, 2 mancas (MZUSP 32629); 5 ♂♂, 5 ♀♀ (UFRGS 5614P); 1 ♂, 2 ♀♀ (UFRGS 5615P).

Diagnosis: The new species is easily distinguished by the shape of the male pleopod 1 exopod: inner margin slightly concave on distal portion, outer margin concave bearing four setae and rounded apex.

Description: Maximum body length 8.0 mm, cephalothorax width 2.0 mm. Light brown color (Fig. 3A). Cephalothorax with irregular unpigmented spots and three large unpigmented areas. Antenna completely pigmented. Pereon with unpigmented longitudinal spots paramedially; epimera 1-4 with one large unpigmented spot and 5-7 with two. Pleonites 1-4 with two large unpigmented spots and pleonite 5 with a large unpigmented area. Telson with three large unpigmented spots. Uropods completely pigmented.

Body (Fig. 3A) convex; dorsal surface smooth and bright, bearing few long piliform scale-setae (Fig. 3B); pleon narrower than pereon with developed epimera; pereonites 1–4 with postero-lateral corners right-angled and posterior margins straight; pereonites 5–7 with postero-lateral corners gradually more acute and posterior margins gradually more arched; pleon epimera 3–5 developed, triangular, acute, and directed backwards. Cephalothorax (Fig. 3C) with linea supra-antennalis centrally bent-downwards and small lamina frontalis; linea frontalis and lateral lobes lacking; clypeus and labrum similar in length; eyes with 15 ommatidia arranged in four rows. One line of *noduli laterales* per side (Fig. 3D, F) with d/c coordinates reaching a maximum on pereonite 4; b/c coordinates gradually decreasing. Telson (Fig. 3G) triangular with lateral margins slightly concave and right-angled apex.

Antennule (Fig. 3H) triarticulate, distal article the longest, with 8 aesthetascs plus apical pair. Antenna (Fig. 3I) when extended posteriorly reaches sixth pereonite, flagellum triarticulate subequals in length; apical organ short, half of the length of the distal article, free sensilla inserted near the base.

Mandibles (Fig. 3J, K) with molar penicil consisting of at least seven branches, pars intermedia with dense tuft of coniform setae, left mandible with three penicils, and right mandible with two penicils. Maxillule (Fig. 3L) medial endite with two penicils, inserted transversely, distal

portion rounded; outer endite with 4+6 teeth, five teeth of inner set cleft, one of them trifid and one simple. Maxilla (Fig. 3M) lateral lobe twice as wide as medial lobe with distal margin sinuous, covered with trichiform setae; medial lobe rounded, covered with trichiform setae. Maxilliped (Fig. 3N) with rectangular base bearing sparse piliform setae; endite rectangular, outer margin sinuous, distal margin rounded and bearing one hook, medial seta overpassing distal margin and a setose ventral sulcus lacking penicil.

Pereopods (Fig. 4A, B) rather slender, piliform setae on all articles, hyaline fringe and sparse setae on merus and carpus; carpus 1 with transverse antenna-grooming brush and one seta with double-serrate apex; ischium 7 triangular, bearing four long setae; dactylus 1–7 with inner claw reaching distal margin of outer claw, dactylar seta long and simple, ungual seta simple, not surpassing inner claw.

Uropod (Fig. 3C) with protopod and exopod grooved on outer margin bearing glandular pores, endopod and exopod inserted at different levels, with few long setae along inner and outer margins; exopod twice as long as endopod, with five setae on apex; endopod with three setae on apex.

Pleopods 1-5 with respiratory areas. Male: Genital papilla (Fig. 3D) with triangular frontal shield distally acute and two subapical orifices. Pleopod 1 (Fig. 3E) exopod heart-shaped, inner margin slightly concave on distal portion, outer margin concave bearing four setae and rounded apex; endopod slightly stout bearing diminute setae along the inner margin and with crenulate plaque. Pleopod 2 (Fig. 3F) exopod triangular, outer margin concave with about three setae; endopod slender and acute apex. Pleopods 3-5 exopod (Fig. 3 G, H, I) rhombus, pleopod 5 exopod outer margin sinuous and bearing three setae.

Remarks: The new species can resembles *Atlantoscia petronioi* and *A. rubromarginata* by the shape of male pleopod 1 endopod but can be distinguished by the number of aesthetascs on antennula (8+2 vs. 12+2 in *A. petronioi* and 6+2 in *A. rubromarginata*), and by the shape of male pleopod 1 exopod, which differs from all other species whitin the genus, including *Atlantoscia* n. sp 1. described

herein (see descriptions and illustrations on Ferrara & Taiti, 1981; Araujo & Leistikow, 1999; Campos-Filho *et al.*, 2012; Campos-Filho *et al.,* 2013).

3.2 Molecular phylogeny of the genus *Atlantoscia*

The final alignment consisted of 33 sequences (11 of *A. petronioi*, 10 of *A. floridana*, five of *Atlantoscia* sp. 1, three of *Atlantoscia* sp. 2, two of *A. ituberasensis*, one of *A. sulcata* and one of *A. rubromarginata*) of 640 bp. In these sequences, the number of variable sites was 235 (36.7%) of which 223 (34.8%) were parsimony informative. The phylogenetic methods (NJ and BI) produced trees with quite congruent topologies, the main difference concerning the support of the clades, which were higher in the BI analysis (e.g., the support of the *Atlantoscia* genus was 1.0 in BI tree and 67% in NJ tree). Hence, only the bayesian analysis is presented (Figure 5). NJ tree can be seen in Figure S1. Posterior probabilities and bootstrap supports are marked at selected nodes, and major clades of *Atlantoscia* are highlighted.

All nominal species of the genus *Atlantoscia* presented high support values (bootstrap of 99% and posterior probability of 1.0), including those described in the present study. Thus, the phylogenetic analysis corroborates the validity of these taxonomic entities. According to the bayesian tree, *A. ituberasensis* and *A. rubromarginata* constitute a more basal clade within the genus. *Atlantoscia sulcata* and *Atlantoscia* sp. 1 compose distinct clades, whereas a larger clade is formed by the species *A. petronioi*, *Atlantoscia* sp. 2 and *A. floridana*.

Individuals of *A. floridana*, *Atlantoscia* sp. 1 and *A. petronioi* were the only ones to present sequence divergence within each group of species. Sequence divergence (K2P) within species of *Atlantoscia* ranged from 0 to 4.4%. *Atlantoscia* sp. 1 presented the highest values of divergence, with a mean divergence of about 2% (Table 2). Sequence divergence among congeneric species ranged from 14.8% (*A. rubromarginata* and *A. ituberasensis*) to 29.3% (*Atlantoscia* sp. 2 and *A. ituberasensis*) (Table 3). The average congeneric distance was of 14.7%. *Atlantoscia ituberasensis* and *A. rubromarginata* presented the highest average genetic divergence when compared to the other

Atlantoscia species (themselves excluded), of 25.9% and 23%, respectively. Between *Atlantoscia* sp. 2 and *Atlantoscia* sp. 1, the two new species described in this study, the divergence value was of 22%.

4. Discussion

In terrestrial isopods, the differences between nominal species are generally small and restricted to a few characters, most of which are subject to intraspecific variation (Poulakakis & Sfenthourakis, 2008). Furthermore, within several species groups, morphological characters do not provide clear taxonomic resolution, so that many changes in the interpretation of nominal species have appeared in the literature (Schmalfuss, 2003). The current study presents the first molecular phylogeny ever made for terrestrial isopods from Neotropical region. We identified the relationships between species of the genus *Atlantoscia* and we tested the value of DNA sequences in the recognition of the described species.

The bayesian tree generated with the COI gene presented a good resolution when distinguishing the species of the genus, since all nominal species of *Atlantoscia* presented high posterior probabilities. Beyond that, reciprocal illumination of morphological characteristics upon a molecular hypothesis supports the proposal of the new species. In other words, the molecular analyses corroborate the current taxonomic systems. According to Costa *et al.* (2007) the barcode region of COI has considerable potential as the foundation for a DNA barcoding identification system for crustaceans, in that a COI-based system will regularly deliver species-level resolution for crustacean lineages (species recognition was straightforward in approximately 95% of the cases).

The topology of the tree produced by the BI analysis supports the existence of at least four different *Atlantoscia* clades. The first clade to emerge (after the outgroups) contains *A. ituberasensis* and *A. rubromarginata*. These are the only species of the genus to present specialized respiratory areas in the pleopods and the only ones to occur solely in the northeast region of the country (Araujo & Leistikow, 1999; Campos-Filho *et al.*, 2013). Subsequently, the clades formed by the species *A. sulcata* and *Atlantoscia* sp. 1 are positioned. The last clade, comprises the species *A. petronioi*,

Atlantoscia sp. 2 and *A. floridana*. *Atlantoscia sulcata* occurs in the southeast region, whereas all of the remaining species (with the exception of *A. floridana*) only have records in the south of Brazil. *Atlantoscia floridana* and *A. petronioi* are morphologically very similar. This great phenotypic similarity combined with the fact that both species are also found in syntopy, makes the distinction between them a challenging task. Nevertheless, the results of the present study (average of divergence of 15% between the species and clades with high support in the BI tree) and recent molecular and ecological evidences have shown that they are, indeed, distinct taxonomic entities (Zimmermann *et al.*, *submitted*).

The mean divergence of congeneric species of *Atlantoscia* was about 15%. Levels of sequence divergence among congeneric species of crustaceans averaged 17.16%, the highest value yet reported for any animal group (Costa *et al.*, 2007). In terrestrial isopods, high genetic divergence values might be linked to the fact that they have restricted gene flow within and between natural populations because they have low dispersal rates, brood their young in a pouch, and require moist microhabitats (Sorensen & Burkett, 1977; Lee *et al.*, 2014). Other authors who have used the COI gene also found high values of genetic divergence in terrestrial isopods. According to Rivera *et al*. (2002), species of *Hawaiioscia* Schultz, 1973 are separated by uncorrected genetic distances of 14.1 to 15.3%. The mean divergence among well-defined *Ligidium* Brandt, 1833 species ranged from 14.4% to 23.3% (Klossa-Kilia *et al.*, 2006). The interspecific comparison between *Porcellio dilatatus* Brandt, 1833 and *P. scaber* Latreille, 1804 revealed a divergence of 16% (Sicard *et al.*, 2014).

According to Costa *et al.* (2007), levels of intra-specific variation in crustaceans averaged 0.46%. However, extremely high values of intra-specific divergence have already been found in terrestrial isopods: the divergence between conspecific *Ligidium beieri* Strouhal, 1928 populations ranged from 5.9% to 15.6% (Klossa-Kilia *et al.*, 2006) and between populations of *Trachelipus aegaeus* (Verhoeff, 1907), these values ranged from 0% to 19% (Kamilari *et al.*, 2014). Deep mitochondrial divergences were also found among groups of individuals of *Spherillo grossus* (Budde-Lund, 1885), with p-distances up to 14% (Lee *et al.*, 2014). In the current study, the intra-specific average distances observed for *A. floridana* and *A. petronioi* were of 0.6 and 0.5%, respectively.

These species showed expected values of intra-specific divergence. *Atlantoscia* sp. 1, on the other hand, showed average intra-specific divergence of 2%, with a maximum of 4.4%.

Atlantoscia ituberasensis and *A. rubromarginata* were the species that presented the highest values of genetic distance concerning the congeneric species. These species are the only ones that possess specialized respiratory areas in the pleopods (Araujo & Leistikow, 1999; Leistikow & Araujo, 2001; Campos-Filho *et al*., 2013). Specialized respiratory areas are a characteristic present in more derived taxa, presumably an adaptation to the life in more xeric environments (Ferrara *et al*. 1990, 1994; Paoli *et al*., 2002; Leistikow & Araujo, 2001). These species are recorded solely from northeast of Brazil, a region well known for its higher temperatures and drier climate (Kottek *et al*. 2006; see Campos-Filho *et al*., 2013 for a species distribution map). Thus, there are two hypotheses which could explain the high genetic divergences observed: they were either promoted by geographic isolation (as it has already been observed for other species of terrestrial isopods, e.g., Klossa-Kilia *et al*., 2006); or *A. ituberasensis* and *A. rubromarginata* do not belong to the genus *Atlantoscia*, something which may be justified by the presence of a unique and derived morphological characteristic in such species. Nonetheless, more studies are necessary in order to clarify this matter.

The differences between nominal species of *Atlantoscia* are generally small and restricted to a few characteristics (basically the shape of male pleopods), most of which are subject to intraspecific variation. These morphological similarities and variations make routine identification difficult for some species (Zimmermann *et al.*, *submitted*). The present study shows that molecular inference is a useful tool, capable of distinguishing between *Atlantoscia* species with quite reliability. DNA barcoding could be helpful in those cases where taxonomy, by itself, is not robust enough to do so. That is, although the task of identifying and describing new species is ultimately achieved through comprehensive taxonomic work, DNA barcodes can significantly facilitate this process (Hajibabaei *et al*., 2007).

As stated by Costa *et al*. (2007), no single approach can provide a definitive conclusion on species boundaries. DNA barcoding is not a substitute for conventional taxonomic approaches, but it

can help in cases where the classical taxonomy does not provide a clear-cut resolution (e.g., cases where current taxonomic systems inappropriately recognize variation as reflecting species status). The recognition of taxonomic boundaries in such cases is always demanding, often subjective, and best pursued through a weight of evidence approach that employs molecular, morphological, and ecological traits to reach a decision.

5. Acknowledgments

6. References

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Table 1: Data on specimens used for this study. Bold letters represent COI sequences utilized for each species of terrestrial isopods in phylogenetic analysis. Record numbers of specimens from scientific collection are listed (Coleção de Carcinologia UFRGS). Sets of letters correspond to Brazilian states: BA - Bahia, RS - Rio Grande do Sul, PR - Paraná, SC - Santa Catarina, SP - São Paulo.

Species	Collection site	Longitude (S)	Latitude (W)	Acession number
Atlantoscia floridana **a**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200831
Atlantoscia floridana **b**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200832
Atlantoscia floridana **c**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200833
Atlantoscia floridana **d**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200834
Atlantoscia floridana **e**	Belém Novo, Porto Alegre, RS	30°12'30''	51°10'12''	KM200835
Atlantoscia floridana **f**	Belém Novo, Porto Alegre, RS	30°12'30''	51°10'12''	KM200836
Atlantoscia floridana **g**	Belém Novo, Porto Alegre, RS	30°12'30''	51°10'12''	KM200837
Atlantoscia floridana **h**	Belém Novo, Porto Alegre, RS	30°12'30''	51°10'12''	KM200838
Atlantoscia floridana **i**	Belém Novo, Porto Alegre, RS	30°12'30''	51°10'12''	KM200839
Atlantoscia floridana **j**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200840
Atlantoscia sp. 1 **a**	Parque St. Hilaire, Matinhos, PR	25°44'42''	48°32'56''	KM200841
Atlantoscia sp. 1 **b**	Belém Novo, Porto Alegre, RS	30°12'30''	51°10'12''	KM200842
Atlantoscia sp. 1 **c**	Belém Novo, Porto Alegre, RS	30°12'30''	51°10'12''	KM200843
Atlantoscia sp. 1 **d**	Belém Novo, Porto Alegre, RS	30°12'30''	51°10'12''	KM200844
Atlantoscia sp. 1 **e**	Belém Novo, Porto Alegre, RS	30°12'30''	51°10'12''	KM200845
Atlantoscia ituberasensis **a**	Santa Cruz da Vitória, BA	15°02'51''	39°47'38''	KM200846
Atlantoscia ituberasensis **b**	Ituberá, BA (UFRGS 4832)	13°43'48''	39°08'53''	KM200847
Atlantoscia sp. 2 **a**	Orleans, SC	28°16'58''	49°23'03''	KM200848
Atlantoscia sp. 2 **b**	Orleans, SC	28°16'58''	49°23'03''	KM200849
Atlantoscia sp. 2 **c**	Orleans, SC	28°16'58''	49°23'03''	KM200850
Atlantoscia petronioi **a**	Rio Grande, RS (UFRGS 5214)	32°00'06''	52°07'08''	KM200851
Atlantoscia petronioi **b**	Rio Grande, RS (UFRGS 5214)	32°00'06''	52°07'08''	KM200852
Atlantoscia petronioi **c**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200853
Atlantoscia petronioi **d**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200854
Atlantoscia petronioi **e**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200855
Atlantoscia petronioi **f**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200856
Atlantoscia petronioi **g**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200857
Atlantoscia petronioi **h**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200858
Atlantoscia petronioi **i**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200859
Atlantoscia petronioi **j**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200860
Atlantoscia petronioi **k**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200861
Atlantoscia rubromarginata **a**	Santa Cruz da Vitória, BA	15°02'51''	39°47'38''	KM200862
Atlantoscia sulcata **a**	Parque das Neblinas, SP (UFRGS 4958)	23°44'01''	45°54'47''	KM200863
Burmoniscus meeusei **a**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200864
Burmoniscus meeusei **b**	Morro Santana, Porto Alegre, RS	30°04'25''	51°07'19''	KM200865
Neotroponiscus littoralis **a**	Ceplac, Ilhéus, BA	14°47'45''	39°17'01''	KM200866
Neotroponiscus littoralis **b**	Ceplac, Ilhéus, BA	14°47'45''	39°17'01''	KM200867

Table 2: Average sequence divergence on citocromo c oxidase subunidade I (COI) gene using (K2P) within species of *Atlantoscia* genus. N/C= not calculated.

Species	Average distance	Min.	Max.
A. floridana	0,006192129	0,00000	0,01134
Atlantoscia sp. 1	0,019998583	0,00000	0,04443
A. ituberasensis	0	0,00000	0,00000
Atlantoscia sp. 2	0	0,00000	0,00000
A. petronioi	0,005594789	0,00000	0,01587
A. rubromarginata	n/c		
A. sulcata	n/c		

Table 3: Sequence divergence on citocromo c oxidase subunidade I (COI) gene between congeneric species of the *Atlantoscia* genus.

	Species group	1	2	3	4	5	6	7
1	*A. floridana*							
2	*Atlantoscia* sp. 1	0,16131						
3	*A. ituberasensis*	0,24332	0,23144					
4	*Atlantoscia* sp. 2	0,16630	0,22056	0,29348				
5	*A. petronioi*	0,15012	0,18891	0,28190	0,16902			
6	*A. rubromarginata*	0,20506	0,23092	0,14798	0,27747	0,24055		
7	*A. sulcata*	0,16513	0,18024	0,19544	0,21292	0,19454	0,21270	
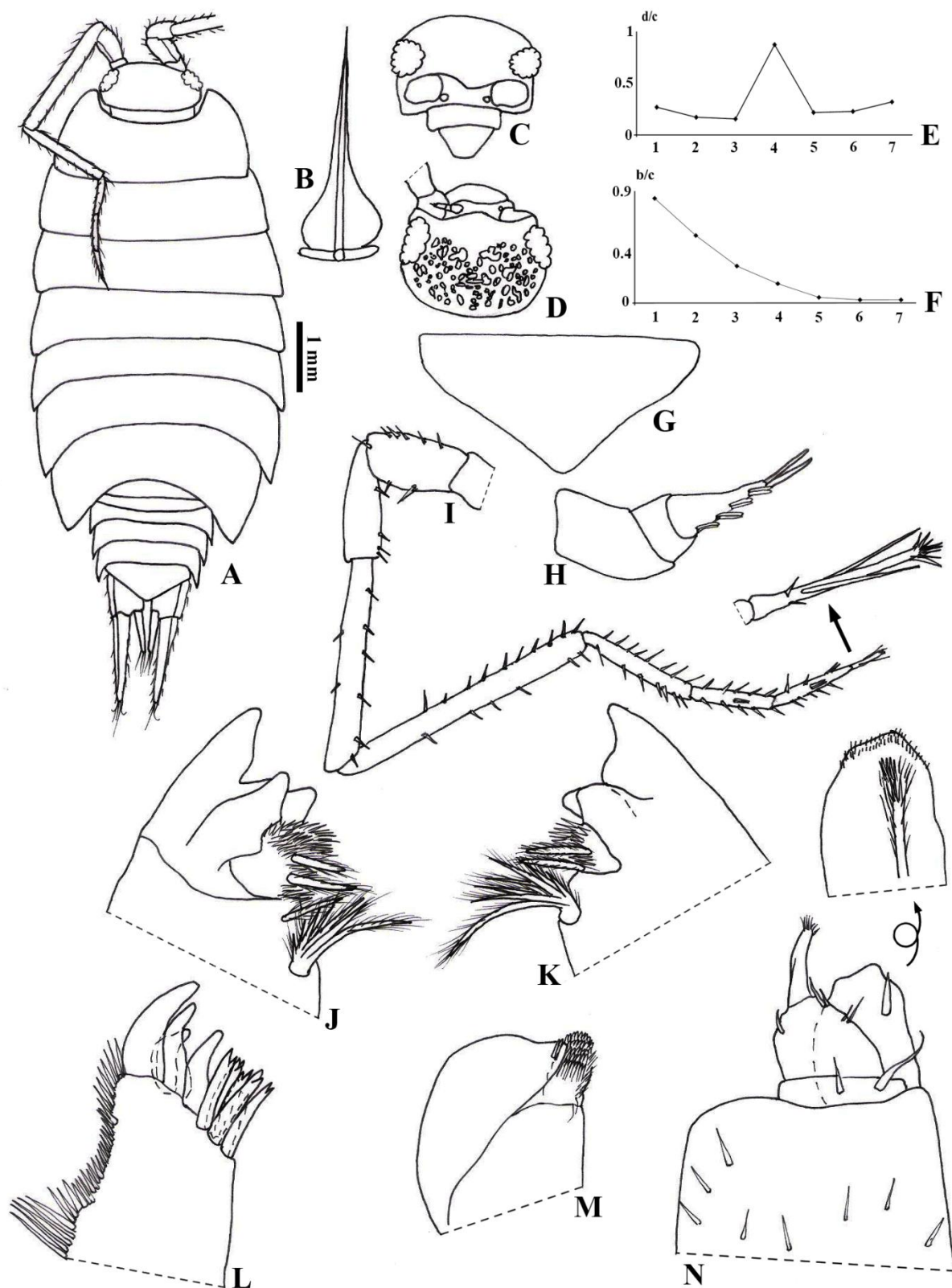
Figure 1: *Atlantoscia* n. sp. 1, male paratype MZUSP 32623. (A) Habitus, dorsal view; (B) scale-seta; (C) cephalothorax, frontal view; (D) cephalothorax, dorsal view; (E) *noduli laterales* coordinates d/c; (F) *noduli laterales* coordinates b/c; (G) telson; (H) antennule; (I) antenna; (J) left mandible; (K) right mandible; (L) maxillula, outer endite; (M) maxilla; (N) maxilliped.
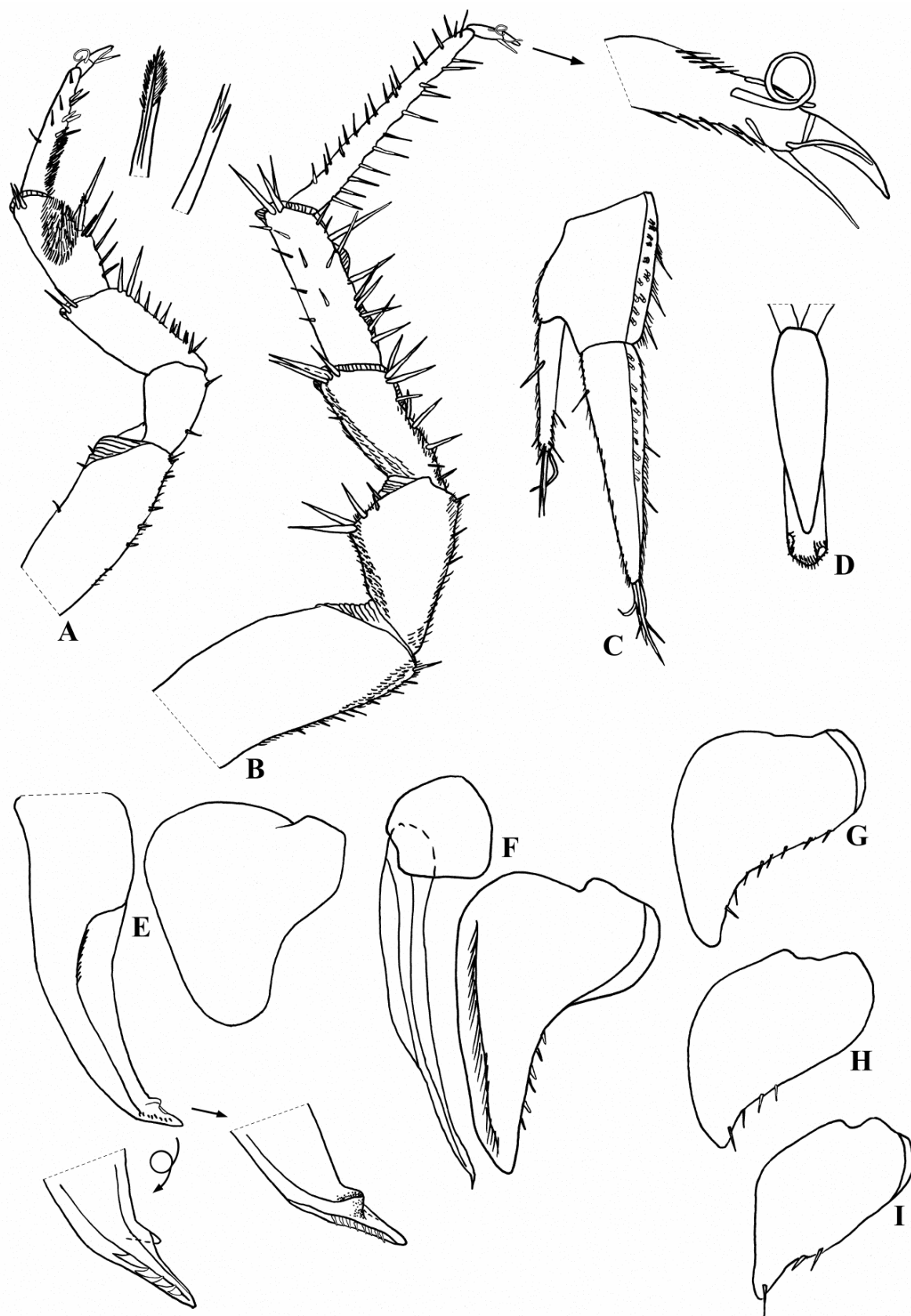
Figure 2: *Atlantoscia* n. sp. 1, male paratype MZUSP 32623. (A) pereopod 1; (B) pereopod 7; (C) uropod; (D) genital papilla; (E) pleopod 1; (F) pleopod 2; (G) pleopod 3 exopod; (H) pleopod 4 exopod; (I) pleopod 5 exopod.
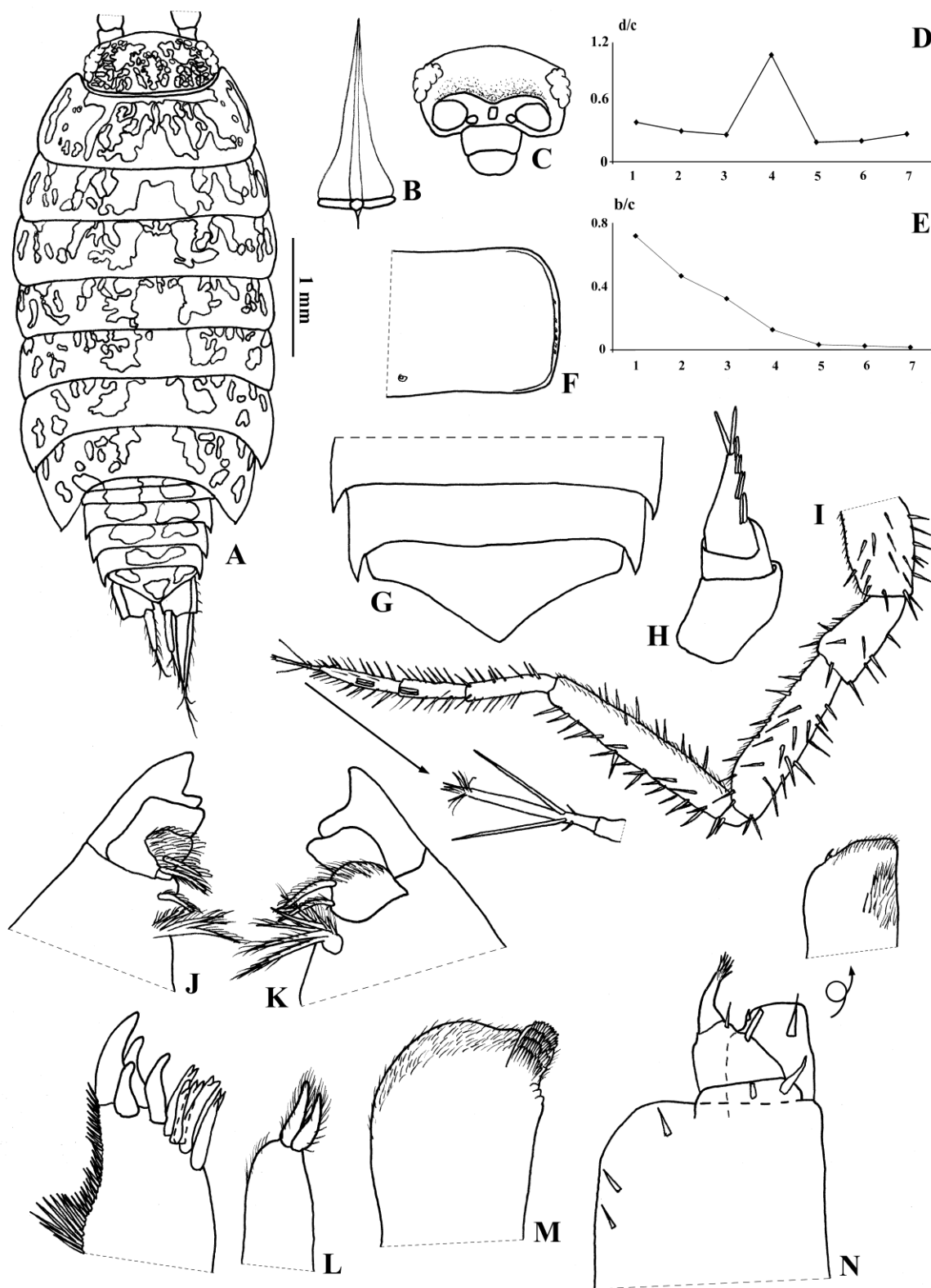
Figure 3: *Atlantoscia* n. sp. 2, male paratype MZUSP 32628. (A) Habitus, dorsal view; (B) scale-seta; (C) cephalothorax, frontal view; (D) *noduli laterales* coordinates d/c; (E) *noduli laterales* coordinates b/c; (F) epimera 4; (G) pleonite 4 and 5, telson; (H) antennule; (I) antenna; (J) left mandible; (K) right mandible; (L) maxillula; (M) maxilla; (N) maxilliped.
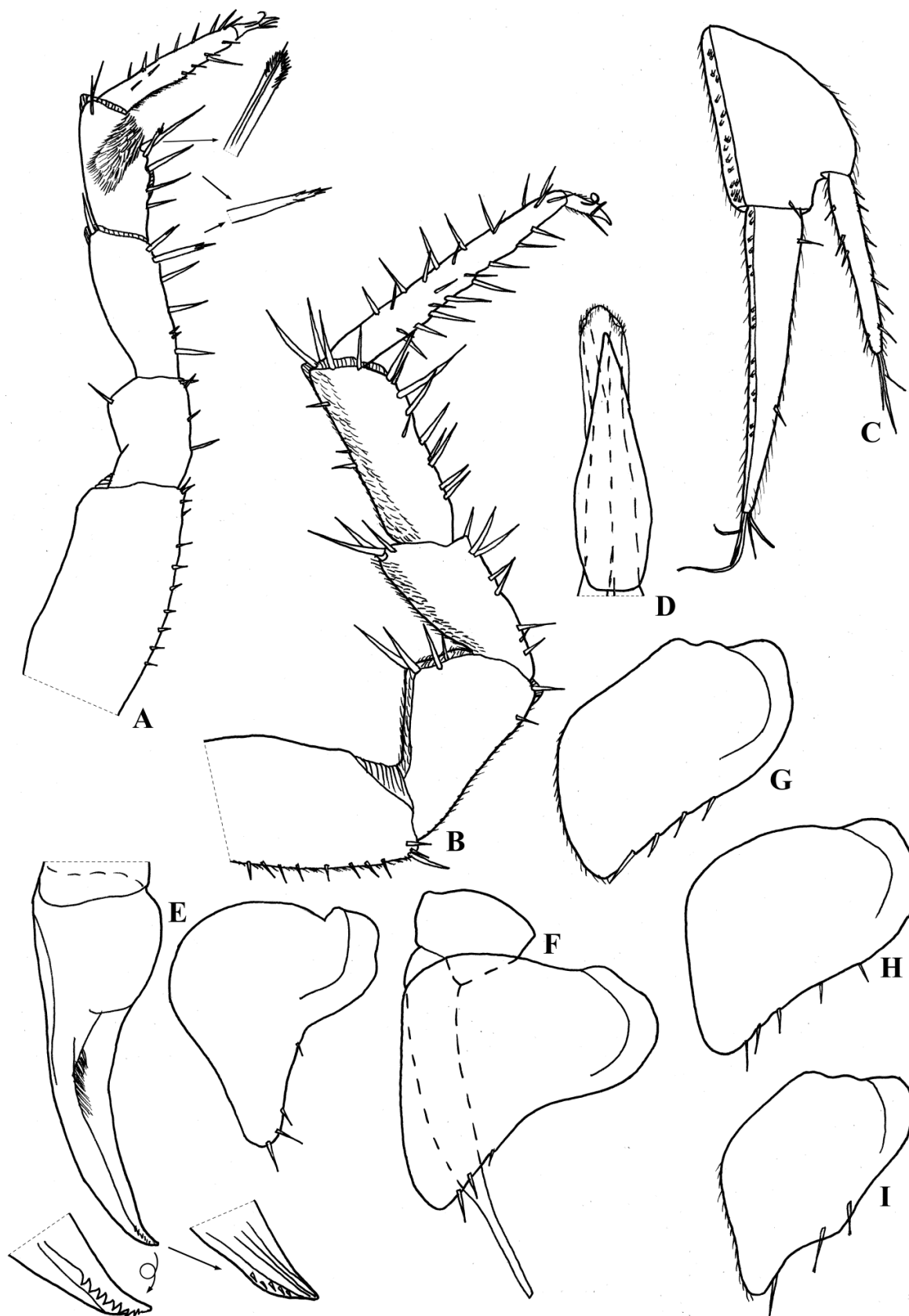
Figure 4: *Atlantoscia* sp. n. 2, male paratype MZUSP 32628. (A) pereopod 1; (B) pereopod 7; (C) uropod; (D) genital papilla; (E) pleopod 1; (F) pleopod 2; (G) pleopod 3 exopod; (H) pleopod 4 exopod; (I) pleopod 5 exopod.
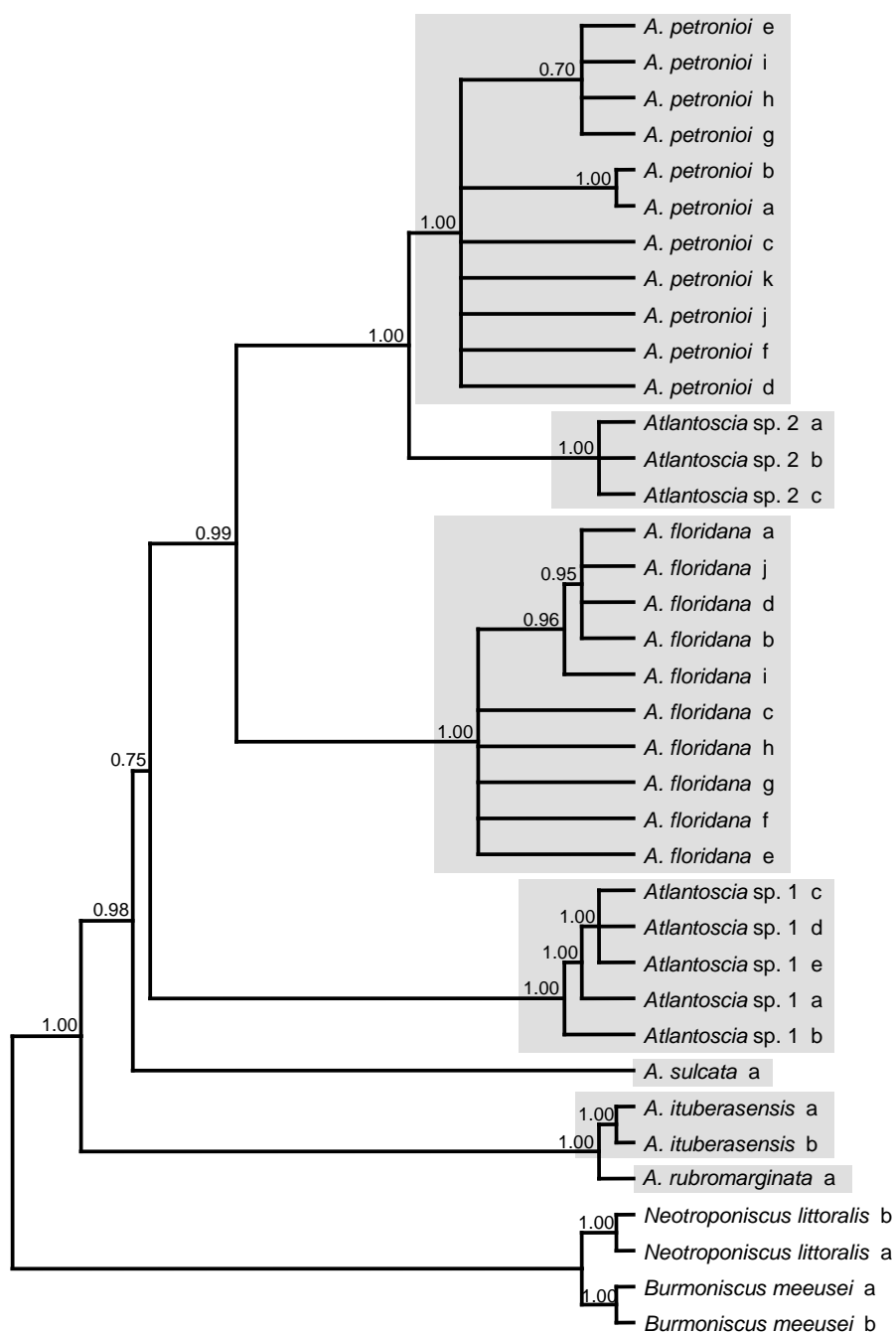
Figure 5: Consensus tree obtained from a 640 bp alignment of COI gene sequences of the *Atlantoscia* species by using Bayesian Inference. Numbers at nodes represent posterior probabilities values (1,000,000 generations). Clades highlighted in the tree correspond to nominal species of *Atlantoscia*.
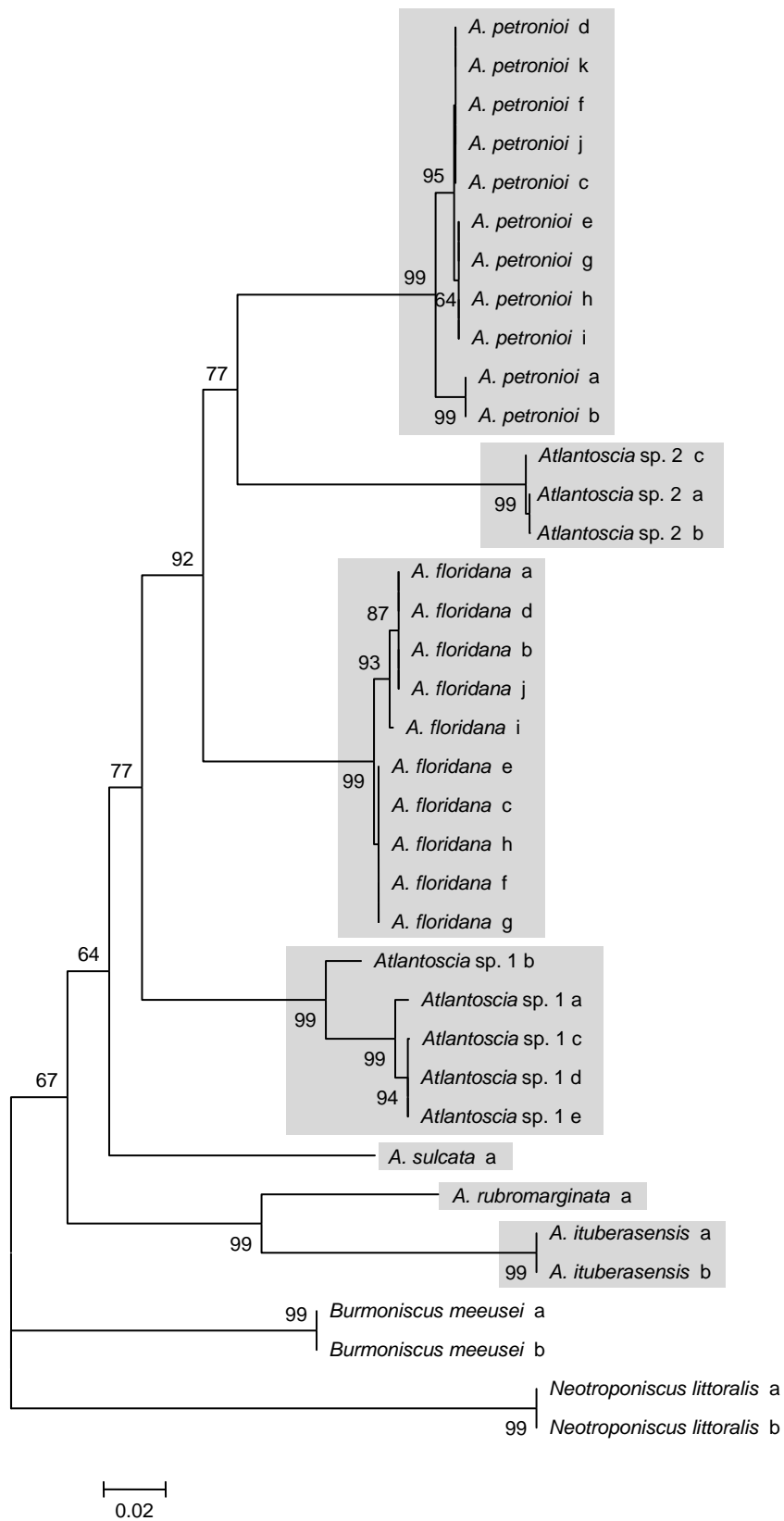
Figure S1: Neighbor-Joining consensus tree obtained for the COI sequence gene of the *Atlantoscia* species. Numbers at nodes represent bootstrap support values (10,000 replicates). Clades highlighted in the tree correspond to nominal species of *Atlantoscia*.

Considerações Finais

As bactérias *Wolbachia* têm sido largamente estudadas e, apesar do relativamente vasto conhecimento existente entre tais bactérias e seus hospedeiros isópodos terrestres na região Peleártica (em especial na Europa), muito pouco é conhecido para a região Neotropical. A presente tese apresenta um esforço inicial para preencher essa lacuna no conhecimento e objetiva conhecer e entender os processos que teriam moldado esta relação simbiótica. Mais especificamente, visou-se investigar aspectos como quais e quantas são espécies de isópodos terrestres infectadas na América do Sul, a diversidade de linhagens de *Wolbachia* presentes nestas espécies, efeito da bactéria sobre a aptidão dos hospedeiros infectados, taxas de transmissão vertical, importância da transmissão horizontal, entre outros. Abaixo são relatados os principais resultados e conclusões, destacando a importância dos achados e enfatizando o que ainda poderia (e deveria) ser feito para dar continuidade às pesquisas.

Primeiramente, no que diz respeito à influência da bactéria sobre a aptidão dos hospedeiros, apesar dos casos já registrados, nos quais a presença de *Wolbachia* confereria vantagens diretas aos mesmos, este não parece ser o caso dos isópodos terrestres. Como observado por outros autores, *Wolbachia* parece ter efeito neutro/deletério na aptidão de *Atlantoscia petronioi*. Interessantemente, foram registradas altas taxas de transmissão vertical nesta espécie, demonstrando que apesar da falta de benefícios óbvios, alguma vantagem possivelmente estaria vinculada à manutenção da infecção em *A. petronioi*. Estudos que avaliem vantagens indiretas da presença da bactéria seriam importantes e essenciais para esclarecer essas questões.

Outra observação que mereceria maior investigação é o fato da ausência de infecção nos indivíduos de *A. floridana* da população de Morro Santana (Porto Alegre), apesar da sua ocorrência sintópica com indivíduos infectados da espécie *A. petronioi*. Alguns experimentos preliminares utilizando TGGE (*Temperature Gradient Gel Electrophoresis*) e não apresentados na presente tese, sugerem a existência de diferenças quali-quantativas nas comunidades bacterianas das duas espécies. Dessa forma, a competição com outras bactérias poderia prevenir a infecção por *Wolbachia* em indivíduos de *A. floridana* dessa população. Todavia, essas são apenas especulações, e experimentos mais consistentes são necessários para confirmar tal hipótese.

Um dos aspectos mais impressionantes deste estudo foi a grande diversidade de linhagens de *Wolbachia* encontrada nas espécies de isópodos terrestres da América do Sul, apesar da baixa prevalência de infecção. Além da variedade de linhagens do supergrupo B, nunca antes a presença de linhagens dos supergrupos A e F havia sido registrada para isópodos terrestres (e nem mesmo para qualquer espécie de crustáceos). Essa incrível diversidade contrasta com o padrão conhecido para as espécies originárias da Europa, nas quais as linhagens de *Wolbachia* são, de modo geral,

altamente convervadas e similares entre si. O porquê dessas diferenças permanece um mistério, assim como a origem de linhagens de supergrupos tão distantes encontradas em *Burmoniscus meeusei* e *Neotroponiscus littoralis* (A e F, respectivamente).

Como já mencionado no terceiro capítulo, a transmissão horizontal da linhagem do supergrupo F a partir dos cupins se mostra como uma possibilidade razoável. Além disso, em estudos recentes foi observado que a presença da linhagem do supergrupo F em *N. littoralis* não é um evento isolado. Foram identificadas mais duas linhagens deste mesmo supergrupo em *Neotroponiscus* (dados não apresentados), uma em espécimes de *N. littoralis* coletados no Rio de Janeiro, e outra linhagem em indivíduos de *N. carolii* coletados na Bahia. Esses dados são muito instigantes e certamente merecem uma análise mais aprofundada, ainda mais se pudermos associar essas linhagens com aquelas presentes nos cupins que vivem em associação com *Neotroponiscus*, comprovando, *in loco*, a transmissão horizontal de *Wolbachia* entre esses grupos de artrópodos.

A filogenia molecular do gênero *Atlantoscia* demonstrou que o gene mitocondrial *COI*, utilizado no *DNA barcoding*, foi eficiente para diferenciar as espécies deste gênero. Uma vez que a inferência molecular corroborou a análise taxonômica clássica, confirmando a validade das espécies nominais de *Atlantoscia*, ela seria uma ferramenta útil para auxiliar nos casos onde os caracteres morfológicos, por si só, não fornecem uma clara resolução. Além disso, a identificação puramente morfológica de espécies similares pode ser difícil aos olhos não treinados, sem contar que a mesma é impossibilitada na ausência de indivíduos do sexo masculino. Considerando que esta é a primeira filogenia molecular para um gênero de isópodos terrestres da região Neotropical, e que novas espécies estão constantemente sendo descritas, a utilização de marcadores moleculares seria altamente recomendada e poderia facilitar em muito na identificação das espécies.

Em suma, apesar das muitas questões que ainda precisam ser respondidas, pode-se dizer que os resultados encontrados na presente tese superaram nossas expectativas. Fica claro que apesar de todo o conhecimento gerado nas últimas décadas de estudos sobre a relação entre *Wolbachia* e seus hospedeiros, dados fascinantes ainda podem ser revelados, principalmente em locais pouco explorados, tal como a região Neotropical. Espera-se que muitos outros estudos ainda sejam realizados, não apenas com isópodos terrestres, mas também com outras espécies de artrópodos e que eles possam desvelar novas facetas e auxiliar no entendimento desta fascinante e intrigante relação simbiótica.