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Research Article

First record of *Wolbachia* in South American terrestrial isopods: Prevalence and diversity in two species of *Balloniscus* (Crustacea, Oniscidea)

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Abstract

Wolbachia are endosymbiotic bacteria that commonly infect arthropods, inducing certain phenotypes in their hosts. So far, no endemic South American species of terrestrial isopods have been investigated for *Wolbachia* infection. In this work, populations from two species of *Balloniscus* (*B. sellowii* and *B. glaber*) were studied through a diagnostic PCR assay. Fifteen new *Wolbachia* 16S rDNA sequences were detected. *Wolbachia* found in both species were generally specific to one population, and five populations hosted two different *Wolbachia* 16S rDNA sequences. Prevalence was higher in *B. glaber* than in *B. sellowii*, but uninfected populations could be found in both species. *Wolbachia* strains from *B. sellowii* had a higher genetic variation than those isolated from *B. glaber*. AMOVA analyses showed that most of the genetic variance was distributed among populations of each species rather than between species, and the phylogenetic analysis suggested that *Wolbachia* strains from *Balloniscus* cluster within Supergroup B, but do not form a single monophyletic clade, suggesting multiple infections for this group. Our results highlight the importance of studying *Wolbachia* prevalence and genetic diversity in Neotropical species and suggest that South American arthropods may harbor a great number of diverse strains, providing an interesting model to investigate the evolution of *Wolbachia* and its hosts.

Keywords: *Wolbachia*, prevalence, diversity, South America, Oniscidea.

Introduction

Wolbachia are maternally transmitted alpha-proteobacteria known to infect a wide range of arthropods and nematodes, where they can be found in either germ line or somatic tissues (Bandi *et al*., 1998; O'Neill *et al*., 1997; Werren *et al*., 1995a). Depending on both bacterial lineage and host, they may have different effects on host reproduction, such as cytoplasmic incompatibility (Breeuwer and Werren, 1990; O'Neill and Karr, 1990), male killing (Hurst *et al*., 1999), parthenogenetic reproduction (Stouthamer, 1997; Zchori-Fein *et al*., 2001) or feminization of genetic males (Bouchon *et al*., 1998; Juchault *et al*., 1994). Therefore, these bacteria may have a strong influence on the evolution of their host populations.

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Terrestrial isopod species (Crustacea, Oniscidea) are widely infected with *Wolbachia* with prevalence reaching ~61% (~36 infected species) in this group (Bouchon *et al*., 2008). Accurate estimates of *Wolbachia* prevalence are difficult to obtain, and both under and/or overestimation may occur. Underestimation may be caused by: (1) incomplete sampling (not all populations are infected), and because (2) infected and uninfected individuals often coexist in the same population (Cook and Butcher, 1999; Hilgenboecker *et al*., 2008). For example, six species tested by Bouchon *et al*. (1998) and showing no evidence of infection were positive in other assays. Furthermore, Verne *et al*. (2011) studied 13 French populations of *Armadillidium vulgare* and found that the prevalence in females ranged from 0% to 100%, and Bouchon *et al*. (2008), in a meta-analysis of infected populations, found a frequency of infected females that ranged from 5% to 74%. In both studies, the authors observed that the overall prevalence of *Wolbachia* was higher than that found in other studies in France with the same species (Cordaux *et al*., 2004; Moret *et al*., 2001;

Rigaud *et al*., 1999). On the other hand, overestimation may result from sampling bias if infected populations are more frequently sampled, and also from false positives in PCR surveys (Li *et al*., 2007).

Due to the non-cultivable nature of *Wolbachia*, the most common method for detecting infection is the enzymatic amplification of one or more *Wolbachia* gene fragments (O'Neill *et al*., 1997). Sequencing of one or several *Wolbachia* genes is applied for genotyping of *Wolbachia* strains, although a multiple-gene sequencing approach would be desirable (Baldo *et al*., 2006b; Paraskevopoulos *et al*., 2006; Ros *et al*., 2009). Previous analyses based on *ftsZ*, *wsp*, (Lo *et al*., 2002; van Meer *et al*., 1999) and 16S rDNA genes (Bandi *et al*., 1999; Vanderkerckhove *et al*., 1999) suggested that current *Wolbachia* strains are structured in eight Supergroups (A-H). Recently, three new supergroups (I, J and K) were proposed based on the analysis of four *Wolbachia* genes (*ftsZ*, *groEL*, *glta* and 16S rDNA) (Ros *et al*., 2009).

Phylogenetic relationships between *Wolbachia* strains are very controversial and could not be well established (Lo *et al*., 2007). It is possible that at least in some cases, exchanges of genetic material (recombination) between *Wolbachia* strains may account for the observed relations between supergroups (Baldo *et al*., 2006a). Recombination is widespread throughout *Wolbachia* genomes, causing some phylogenetic uncertainties. For example, Supergroup G, containing strains infecting spiders, seems to be the result of recombination between strains of Supergroups A and B (Baldo and Werren, 2007). However it is well known that the phylogenetic relationships of *Wolbachia* do not mirror those of its hosts since numerous host shifts have been reported (Riegler and O'Neill, 2006).

To date, almost all *Wolbachia* strains from terrestrial isopods belong to Supergroup B, based on 16 rDNA and *ftsZ* sequences and corroborated by *wsp* and *groEL* phylogenies (Bouchon *et al*., 1998; Cordaux *et al*., 2001; Baldo *et al*., 2006b; Wiwatanaratanabutr *et al*., 2009). In Supergroup B, phylogenies suggested that *Wolbachia* strains from terrestrial isopods form a polyphyletic clade, even though the majority of strains group in the *Oni*clade (Bouchon *et al*., 1998; Cordaux *et al*., 2001; Wiwatanaratanabutr *et al*., 2009).

Although terrestrial isopods have long been known to be infected (Legrand and Juchault, 1970), no endemic South American species has been investigated up to now for prevalence and genetic diversity of *Wolbachia*. *Balloniscus sellowii* is a species with wide distribution, occurring from southern Brazil to Uruguay and Argentina (Lemos de Castro, 1976), where it is often associated with anthropized environments and exotic forests (Araujo *et al*., 1996). On the other hand, *B. glaber* (Araujo and Zardo, 1995), whose distribution overlaps with that of *B. sellowii*, is endemic and restricted to the southernmost state of Brazil, Rio Grande do Sul, where it occurs associated with remnants of the Brazilian Atlantic Forest. Due to deforestation, fragmentation and replacement by exotic species in this biome (Morellato and Haddad, 2000), *B. glaber* is susceptible to extinction (Quadros *et al*., 2009).

Here, we present the first description of *Wolbachia* infection in two South American species of terrestrial isopods, specifically in *Balloniscus sellowii* and *B. glaber*. Moreover, *Wolbachia* sequences were shown to exhibit a high level of genetic variation within and between their host species. There is evidence of cross-specific transmissions between species and the evolutionary implications of these observations are discussed.

Material and Methods

Sample collection

Individuals of *B. sellowii* and *B. glaber* were hand collected from soil, placed in 100% ethanol and kept refrigerated until DNA extraction. Nine populations of *B. glaber* and thirteen populations of *B. sellowii* were sampled in the coastal plain of Rio Grande do Sul, Brazil (Figure 1). This area is an elongate (620 km) and wide (up to 100 km) physiographic province which covers about 33,000 km^2 (Tomazelli *et al*., 2000).

DNA extraction

Total genomic DNA was obtained by the Chelex® (BioRad) method from different parts of the body including

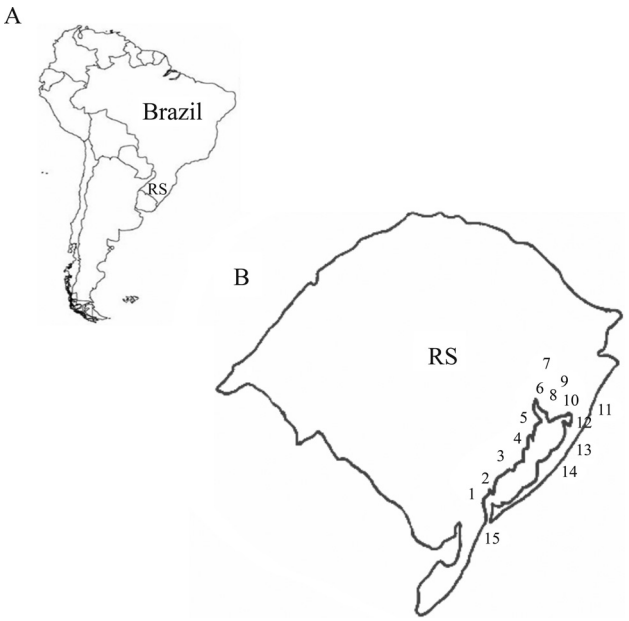

Figure 1 - Study area (RS: Rio Grande do Sul; PL: Patos lagoon). Populations; PEL (1) (Pelotas); CZ3 (2) (Colônia Z3); SLS (3) (São Lourenço do Sul); TAP (4) (Tapes); BRI (5) (Barra do Ribeiro); ABE (6) (Águas Belas); CSU (7) (Caxias do Sul); POA (8) (Porto Alegre); MSA (9) (Morro Santana); GLO (10) (Glorinha); CID (11) (Cidreira); PAL (12) (Palmares do Sul);. MOS (13) (Mostardas); TAV (14) (Tavares); CAS (15) (Cassino).

reproductive (ovaries and testis), nerve and muscular tissue of fixed individuals . Dissection procedures were performed under sterile conditions as previously described (Bouchon *et al*., 1998).

Assay for *Wolbachia*

The presence of *Wolbachia* was detected by PCR with *Wolbachia*-specific primers for 16S rDNA (O'Neill *et al*., 1992). Failure of amplification with general 16S rDNA primers could be due to either: (i) absence of *Wolbachia*, (ii) failure in DNA extraction procedure, and/or (iii) incorrect concentration of DNA solutions (Werren *et al*., 1995a). In order to control for the last two possibilities, we tested whether the samples scored as negative for *Wolbachia* would result in positive amplification of the host mitochondrial cytochrome oxidase subunit I gene (*COI*) (Folmer *et al*., 1994). Samples yielding a product of expected size were considered to be true negatives for the *Wolbachia* assay.

Additionally, we tested four other *Wolbachia* genes (*groEL, dnaA*, *ftsZ* and *wsp*) using PCR, and each of them showed different results for *Wolbachia* detection. For example, in one assay out of 20 positives for the 16S rDNA gene, we observed only ten, six, four and two positives for *groEL, dnaA*, *ftsZ* and *wsp* genes, respectively. Based on these findings we concluded that amplification of the 16S rDNA seems to be the most sensitive for *Wolbachia* detection in *Balloniscus* species by means of a PCR assay. Furthermore, we tested five and 10 individuals of *Armadillidium vulgare* and *A. nasatum*, respectively, for all genes cited above. These are exotic Palearctic species forming sympatric populations with those of *Balloniscus*. Variations in prevalence were checked by logistic regressions using JMP 5.0.1 software (SAS Institute).

PCR methods, purification and sequencing

We amplified most of the *Wolbachia* 16SrDNA gene 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' (O'Neill *et al*., 1992), which yield a product of around 900 bp. PCR was carried out in a volume of 25 μL, using 12.5 ng of total DNA, 0.125 U of Go*Taq* (Promega), 1x *GoTaq* buffer (Promega), 5 μM of each primer, and 0.2 mM of dNTPs. PCR cycling conditions were 35 cycles (1 min at 95 °C, 1 min at 50.6 °C, 1 min at 72 °C), including an initial denaturing step of 95 °C for 2 min and a final extension step of 72 °C for 5 min. To check PCR success and size of the amplified DNA, 5 μL of the reaction product was run on 1.5% agarose gel. PCR products were purified using Exosap-IT (Amersham Biosciences). Sequencing was done using BigDye v3.1 and an ABI 3130 Genetic Analyzer (Applied Biosystems). All different strains were deposited in the GenBank -EMBL database under the accession numbers GQ229434-GQ229446 and GQ229448-GQ229450.

Sequence polymorphism and phylogeny

A total of 77 16S rDNA sequences, representing almost all *Wolbachia* supergroups were aligned using Clustal X (Thompson *et al*., 1997). The alignment was visually inspected using the BioEdit program (Hall, 1999). Exceptions were *Wolbachia* 16S rDNA sequences of Supergroups G and H. As sequences from Supergroup G probably resulted from a recombination event between strains of Supergroups A and B (Baldo and Werren, 2007), the inclusion of sequences from these supergroups resulted in phylogenetic trees with very low support values (data not shown). Concerning sequences from Supergroup H (*Zootermopsis angusticollis* - AY764279 and *Z. nevadensis* - AY764280) a different portion of the 12S gene was amplified and these sequences did not align with those sequenced in this study.

Two 16S rDNA sequences from *Rickettsia ricketsii* and *Anaplasma marginale* were included as outgroups. Alignment was improved by checking the secondary structure of 16S rDNA sequences according to the RDP database. As the current strain definition in *Wolbachia* involves data from multiple genes we did not consider the 16S lineages found in this study as representing different bacterial "strains". Anyway, for naming purposes two lineages were considered identical only if they had identical nucleotide sequences. Presence of recombination was checked using RDP version 2.0 (Martin *et al*., 2005), since recombination may occur in individuals infected with multiple *Wolbachia* strains (see Jiggins *et al.*, 2001 for a review). We used the Arlequin 3.1 program (Excoffier *et al*., 2005) to assess how much of the total genetic variation (measured by Φ_{ST}) was partitioned between the two species through AMOVA (Excoffier *et al*., 1992).

The phylogeny of all *Wolbachia* sequences was estimated using the Bayesian method implemented in Beast version 1.4.7 software (Drummond and Rambaut, 2007), in which the tree-space and the parameter space are analyzed simultaneously running a MCMC for 100,000,000 steps based on the GTR+Γ model, on a Yule speciation process, and assuming a lognormal relaxed molecular clock (Drummond *et al*., 2006). All other priors were set to the default values. The monophyly of *Wolbachia* lineages from *Balloniscus* was tested through the Bayes factor (BF) (Suchard *et al.*, 2005) by comparing an unconstrained topology with that generated with the same search strategy described above, but enforcing a monophyletic group formed by all lineages found in *Balloniscus*. Results are expressed as $\log_{10}$BF. Values of $\log_{10}BF = 2$ or higher are considered significant evidence for a given hypothesis (Kass and Raftery, 1995).

Finally, a median-joining network (Bandelt *et al.*, 2000) was constructed using the Network version 4.610 software to check the number of mutated nucleotide positions defining each grouping, as well as to infer the genetic

diversity within the 16S rDNA sequences from Supergroup B.

Results

Wolbachia prevalence in *B. glaber* and *B. sellowii*

In all, 265 individuals of *B. sellowii* were sampled in 13 populations with 42 of them being infected, while for *B. glaber* 254 individuals were sampled in nine populations with 99 individuals being infected (Table 1). There was no significant difference in population sex ratio for either species (Fisher's exact test, p = 0.5206). The percentages of males and females were 34% and 66% and 37% and 63% in *B. selowii* and *B. glaber*, respectively. No intersexual individual was found in either species. *B. sellowii* showed no differences of prevalence between males (13%) and females (17%) (Logistic regression, Wald test, p = 0.9998), while *B. glaber* showed a higher *Wolbachia* prevalence in females (45%) than in males (23%); (Logistic regression Wald test; p < 0.001). On average, *B. sellowii* also showed lower infection rates than *B. glaber* (16% and 37%; Fisher's exact test, p < 0.001), mainly due to the higher frequency of infected females in *B. glaber* (nested logistic regression, Wald test, p < 0.001) (Table 1). For each species, the prevalence varied widely in different populations (logistic regression, Wald test, p < 0.001) ranging from 0% to 52.6% in *B. selowii* and from 0% to 87.5% in *B. glaber*. *Wolbachia* was found in seven out of nine populations of *B. glaber* and in nine out of 13 populations of *B. sellowii* suggesting that *Wolbachia* prevalence is not different between these species (Fisher's exact test; p = 0.65). Considering both species, no infected individuals were found in four (ABE, 6; PEL, 1; PAL, 12 and CSU, 7) out of 15 populations. *B. sellowii* and *B. glaber* were sympatric in two of all uninfected populations (ABE, 6 and PEL, 1), while in the remaining two (PAL, 12 and CSU, 7) only *B. sellowii* individuals were observed.

Genetic variation among *Wolbachia* 16S rDNA sequences from *Balloniscus*

A total of 35 sequences of *Wolbachia* 16S rDNA from *Balloniscus* were obtained. No recombination event was detected among the sequences. The general level of genetic diversity is presented in Table 2. By any measure, *Wolbachia* sequences from *B. sellowii* had a higher genetic variation when compared to those isolated from *B. glaber*. AMOVA estimates were that 25.61% of the total genetic variance is contained at the between-species level, while an additional 60.15% is distributed among populations of each species. The within-population level accounts for the remaining 14.24% of the genetic variance. The genetic diversity found in *Wolbachia* 16S rDNA sequences from *Balloniscus* was higher than that found in sequences obtained from species belonging to the genus *Armadillidium*,

Table 1 - Prevalence of *Wolbachia* in *B. sellowii* and *B. glaber* populations.

	B. sellowii					*B. glaber*				
Locality	N (F/M)	Strain	IF	IM	II	N (F/M)	Strain	IF	IM	II
ABE (6)	7/12	-	0	0	0	26/21	-	0	0	0
BRI (5)	20/6	*w*Sel1	3	2	5	13/8	*w*Gla1	11	4	15
CAS (15)	11/5	*w*Sel2	1	0	1	18/12	*w*Gla1	11	4	16
CID (11)	13/11	*w*Sel7	4	1	5	26/6	*w*Gla3	24	4	28
CSU (7)	8/5	-	0	0	0	-	-	-	-	-
CZ3 (2)	25/13	*w*Bal2 *w*Sel6	4*	1*	5*	15/17	*w*Gla1	12	8	20
GLO (10)	11/5	*w*Sel3 *w*Sel7	2*	0*	2*	-	-	-	-	-
MOS (14)	-	-	-	-	-	10/5	*w*Gla2	2	2	4
MSA (9)	-	-	-	-	-	33/11	*w*Bal2	5	0	5
PAL (12)	14/8	-	0	0	0	-	-	-	-	-
PEL (1)	11/5	-	0	0	0	6/5	-	0	0	0
POA (8)	20/4	*w*Sel4 *w*Sel5	8*	1*	9*	-	-	-	-	-
SLS (3)	13/6	*w*Sel8 *w*Sel9	8*	2*	10*	-	-	-	-	-
TAP (4)	12/6	*w*Sel9 *w*Sel10	0*	4*	4*	-	-	-	-	-
TAV (14)	10/4	*w*Bal1	0	1	1	13/9	*w*Bal1	7	0	7
Total	175/90		30	12	42	160/94		77	22	99

N = Number of individuals tested (Females; Males); IF: Number of infected females; IM: Number of infected males; II: Number of infected individuals. *: Overall prevalence (individuals may harbor one or both sequences found in these populations) -: Species not found; Populations: PEL (1) (Pelotas); CZ3 (2) (Colônia Z3); SLS (3) (São Lourenço do Sul); TAP (4) (Tapes); BRI (5) (Barra do Ribeiro); ABE (6) (Águas Belas); CSU (7) (Caxias do Sul); POA (8) (Porto Alegre); MSA (9) (Morro Santana); GLO (10) (Glorinha); CID (11) (Cidreira); PAL (12) (Palmares do Sul);. MOS (13) (Mostardas); TAV (14) (Tavares); CAS (15) (Cassino).

Table 2 - *Wolbachia* diversity and polymorphism of 16S rDNA in *Balloniscus* species and European isopods.

	Balloniscus	*Armadillidium*
Number of polymorphic sites	*B. sellowii* (24)	*Armadillidium* genus[1] (15)
	B. glaber (9)	All sampled European species[2] (34)
	Balloniscus genus (33)	
Nucleotide diversity (π)	*B. sellowii* (0.008)	*Armadillidium* genus[1] (0.002)
	B. glaber (0.005)	All sampled European species[2] (0.013)
	Balloniscus genus (0.010)	

[1]*A. vulgare* Angouleme (France) AJ223238, *A. vulgare* Niort (France) X65669, *A. vulgare* Dunasziget (Hungary) AJ306311, *A. nasatum* Mignaloux (France) AJ223239, *A. nasatum* Barra do Ribeiro/RS (Brazil) GQ 229450, *A. album* Yves (France) AJ223240.
[2]*A. album* Yves (France) AJ223240, *A. nasatum* Mignaloux (France) AJ223239, *A. vulgare* Angouleme (France) AJ223238, *A. vulgare* Niort (France) X65669, *A. vulgare* Dunasziget (Hungary) AJ306311, *Cylisticus. convexus* Avanton (France) AJ001602, *C. convexus* Tatabanya (Hungary) AJ306312, *Porcellio scaber* Ahun (France) AJ001608, *P. scaber* Dunasziget AJ306307, *P. spinicornis* Quincay (France) AJ001609, *P. dilatatus* St. Honorat Island (France) X65673, *P. dilatatus* Tatabanya (Hungary) AJ306314, *Chaetophiloscia elongata* Celle sur Belle (France) AJ223241, *Helleria brevicornis* Bastia (France) AJ001603, *Haplophthalmus danicus* Quincay (France) AJ001604, *Ligia oceanica* Angoulins (France) AJ001605, *Oniscus asellus* Quincay (France) AJ001606, *Philoscia muscorum* Quincay (France) AJ001607, *Porcellionides pruinosus* AJ 223242, *P. pruinosus* AJ133196 , *Trachelipus atzeburgii* Tatabanya (Hungary) AJ306315, *T. ratzeburgii* Dunasziget (Hungary) AJ306309, *T. politus* Tatabanya (Hungary) AJ306313, *T. rathkii* Dunasziget (Hungary) AJ306310, *H. riparius* Dunasziget (Hungary) AJ306308.

and was comparable to that found in sequences obtained from all species of European terrestrial isopods together (Table 2).

Wolbachia 16S rDNA sequence diversity and distribution

We found 15 new *Wolbachia* sequences in the two *Balloniscus* species studied here. Among these, 10 were associated exclusively with *B. sellowii* and named *w*Sel1, *w*Sel2, *w*Sel3, *w*Sel4, *w*Sel5, *w*Sel6, *w*Sel7, *w*Sel8, *w*Sel9 and *w*Sel10, and three sequences were associated exclusively with *B. glaber* and named *w*Gla1, *w*Gla2 and *w*Gla3. Furthermore, two sequences were shared by both species: *w*Bal1 and *w*Bal2. The 16S rDNA sequence of the *Wolbachia* found in one individual of *A. nasatum* sampled in BRI (5) (*w*NasBRI) differed from those found in European populations by a single 1 bp indel. Noteworthy, five out of nine infected populations (CZ3, 2; GLO, 10; POA, 8; SLS, 4 and TAP, 4) hosted two different *Wolbachia* 16S rDNA sequences, although no evidence of co-infections in a single individual was found.

Phylogenetic relationship of 16S rDNA *Wolbachia* sequences

The inferred phylogenetic relationship among *Wolbachia* 16S rDNA sequences found in this study and from other hosts is shown in Figure 2 together with the Bayesian posterior clade probabilities (PCP). This analysis suggested that *Balloniscus Wolbachia* sequences group in Supergroup B, in agreement with previous results obtained from other species of terrestrial isopods (Bouchon *et al*., 1998, Cordaux *et al*., 2001, Wiwatanaratanabutr *et al*., 2009). However, they fell outside the classical terrestrial isopod subclade named *Oni* clade (which was recovered with Bayesian PCP of 1.00 in this study) (Cordaux *et al*., 2001).

Wolbachia sequences from *Balloniscus* grouped in three different positions in the phylogenetic tree (Figure 2): twelve *Wolbachia* sequences (*w*Sel1, *w*Sel2, *w*Sel3, *w*Sel4, *w*Sel5, *w*Sel6, *w*Sel7, *w*Sel8, *w*Sel9, *w*Sel10, *w*Gla3 and *w*Bal2) formed a cluster with Bayesian PCP 1.00; alternatively, *w*Gla2 and *w*Bal1 formed a small cluster with Bayesian PCP 0.99; while *w*Gla1 did not group with any other sequence with high support. Interestingly, the *Wolbachia* sequences obtained from *Balloniscus* species did not form a monophyletic group, which could be indicative of multiple infections occurring throughout the history of these species.

When compared to an alternative topology where all *Wolbachia* 16S rDNA sequences from *Balloniscus* were forced into a monophyletic clade the unconstrained topology (non-monophyly hypothesis) was supported by the Bayes factor analysis by a $\log_{10}$BF of 2.033, which is considered to be strong evidence in favor of a hypothesis. This value can be understood intuitively as a support of ~108:1 in favor of the unconstrained topology. The scenario of multiple infections was also supported in the median-joining network (Supplementary Material Figure S1), since *Wolbachia* 16S rDNA sequences from *Balloniscus* were mixed with other sequences found in different species, in such a way that forcing their monophyly would necessarily invoke a number of recurrent mutations. Notably, none of the analyses suggest that the sequences infecting each *Balloniscus* species form a monophyletic group, even when only species-specific *Wolbachia* sequences are taken into account.

Discussion

Wolbachia is a maternally inherited endosymbiont, common to several species of terrestrial isopods (Bouchon

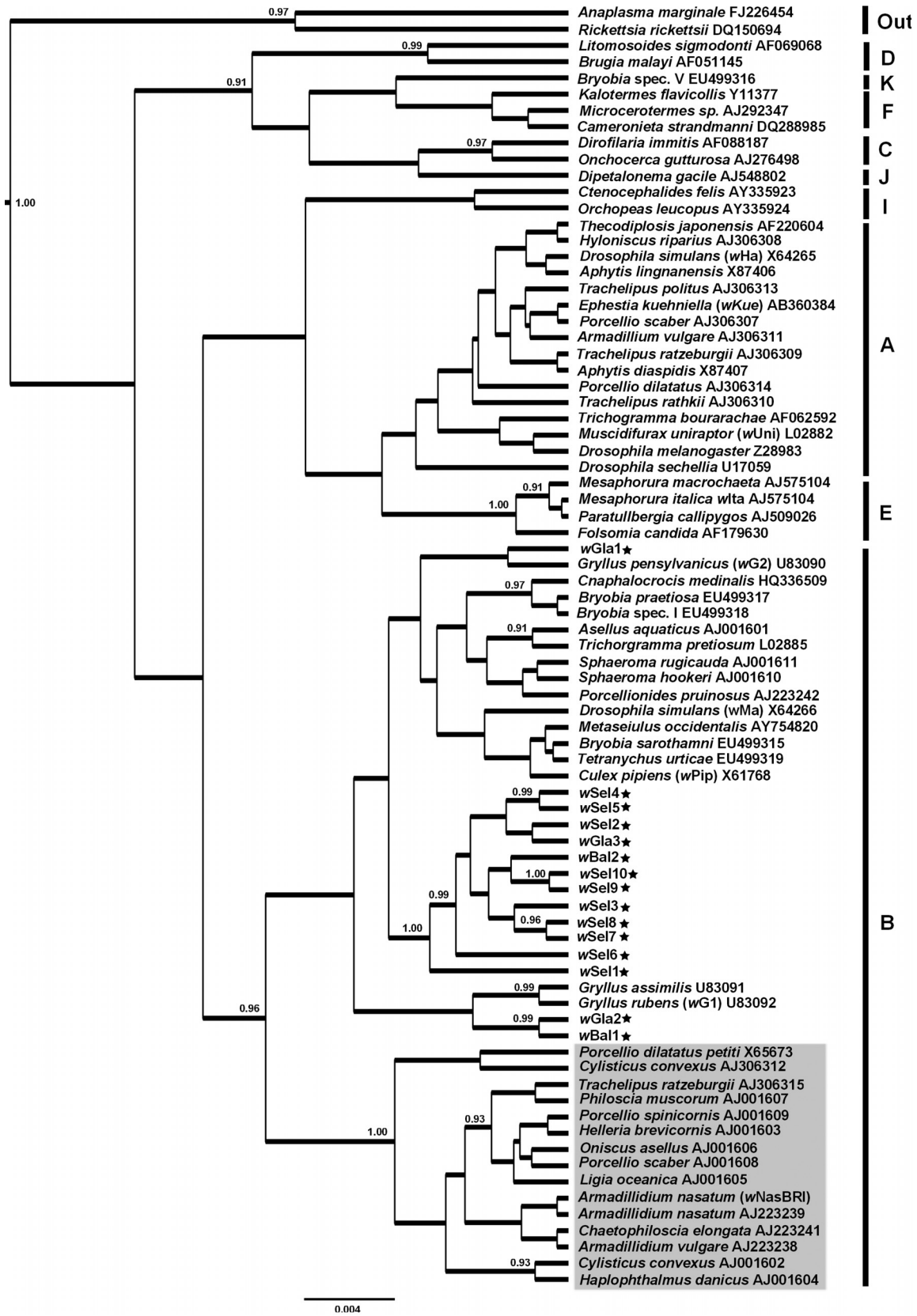

Figure 2 - Bayesian tree constructed based on partial 16S rDNA sequences of *Wolbachia* from *Balloniscus* species (denoted with stars) and another 16S rDNA from *Wolbachia* supergroups. Bayesian PCP values are shown (only those above 0.90). Names the host arthropod species followed by *Wolbachia* strain name and an accession number denotes the specific *Wolbachia* strain. Letters A-F denote *Wolbachia* supergroups; out: outgroup. Grey square: *Oni* clade.

et al., 1998, 2008; Cordaux *et al*., 2001, Nyrö *et al*., 2002; Ben Afia Hatira *et al*., 2008; Wiwatanaratanabutr *et al*., 2009) and infecting North African, North American, Southeast Asia and European populations. This study presents the first record of *Wolbachia* occurrence in South American species of terrestrial isopods, and shows the highest sequence diversity previously found among terrestrial isopods for the *Wolbachia* 16S rDNA gene.

Wolbachia prevalence

Overall, *B. sellowii* was, on average, less infected than *B. glaber*. A possible explanation for this is an older co-evolutionary history with *B. sellowii*, which therefore would have more protection mechanisms against *Wolbachia* infection. This explanation is supported by our phylogenetic analyses, indicating that *B. sellowii* is a less fortuitous host, being infected by more genetically related lineages of *Wolbachia*.

For two populations (ABE, 6 and PEL, 1), where *B. sellowii* and *B. glaber* were sympatric, we could not detect infection by *Wolbachia* in any specimen. In addition, *Wolbachia* was not found in two other *B. sellowii* populations (PAL, 12 and CSU, 7). At least two non-exclusive hypotheses can explain this pattern. Either *Wolbachia* infection did not reach these populations or some evolutionary mechanisms are keeping *Wolbachia* prevalence at a very low level (decreasing the probability of sampling *Wolbachia* infected specimens). In the first case, the absence of migration between infected and uninfected population may prevent infection. The second case involves several potential factors. For example, environmental conditions (Keller *et al*., 2004), deficiency in vertical transmission (Rigaud and Juchault, 1992), competition between *Wolbachia* strains or with other elements (parasites or genetic factors), time of the *Wolbachia* invasion (Shoemaker *et al.*, 2003), or stochastic demographic factors (Rigaud and Juchault, 1992, Jansen *et al*., 2008) which can slow down the spread of *Wolbachia* within and across populations, or even exclude the reproductive parasites from some host populations. Horizontal transfers from one species to the other, although rare when compared to migrations, can partly counterbalance some of the factors previously discussed. An interesting observation was the high variation in *Wolbachia* prevalence resulting from intragenomic conflict between different sex distorters reported in some European populations (Bouchon *et al*., 2008). As this was the first attempt to detect *Wolbachia* infection in Brazilian Oniscidea, understanding the role of such numerous factors will require much future efforts.

Genetic diversity and phylogeny of *Wolbachia* sequences

Some recent studies used a multiple-gene PCR and sequencing approach for the detection and characterization of *Wolbachia* lineages (Ros *et al*., 2009). Even though our findings are only based on the *Wolbachia* 16S rDNA gene, this marker has been widely used to detect the presence of *Wolbachia* and assess the level of polymorphism of its lineages (*e.g.* Werren *et al*., 1995b; Bouchon *et al*., 1998; Vandekerckhove *et al*., 1999; Rowley *et al*., 2004; Bordenstein and Rosengaus, 2005). However, because of the limitations of characterizing *Wolbachia* strains based on only one gene we did not consider sequences as true "strains", and other assays must be performed to resolve this issue.

Nonetheless, because the 16S rDNA gene is less polymorphic than other markers, such as *wsp* (Schulenburg *et al.*, 2000), the genetic diversity of the sequences found in *Balloniscus* is very impressive and higher than that observed in *Wolbachia* 16S lineages isolated from European species. For example, the mean *Wolbachia* sequence divergence found for the genus *Balloniscus* was comparable to that for *Wolbachia* sequences from all species of European terrestrial isopods (Table 2). Also, the AMOVA results suggested that the genetic diversity of the parasite is not obviously limited by the specific barriers of its host. Processes like horizontal *Wolbachia* transmission between *Balloniscus* species and/or multiple infections from other arthropod hosts may play some role in preventing a clear relationship between the phylogeny of the host species and *Wolbachia*. These findings would suggest a different co-evolutionary history between terrestrial isopods and *Wolbachia* from South America and Europe.

The phylogenetic analysis showed that *Wolbachia* sequences from *Balloniscus* were grouped outside the *Oni* clade, corroborating previous findings of terrestrial isopod species (one in Europe and two in Asia) where *Wolbachia* lineages were also found outside this clade (Cordaux *et al*., 2001; Nyrö *et al*., 2002; Wiwatanaratanabutr *et al*., 2009). A *Wolbachia* sequence from *A. nasatum*, a European species which was introduced to South America, was found in one individual of BRI (5). This sequence was identical to the one found in French populations of this species. Studies from other introduced species also showed that their *Wolbachia* were carried during introduction (Zimmermann *et al*., unpublished data). These results suggest that *Wolbachia* followed independent evolutionary trajectories in South America and Europe and corroborates the idea that *Wolbachia* spread differently within each host clade, according to geographic regions (Wiwatanaratanabutr *et al*., 2009).

Concerning the origins of the *Wolbachia* in *Balloniscus*, the monophyly of these sequences has not been supported based on Bayes Factor (BF). The BF is calculated by integrating the parameter space and tree space and provides an intuitive way of contrasting alternative hypotheses in a Bayesian framework (Suchard *et al*., 2005). However, estimating BF based on posterior samples drawn from MCMC phylogenetic algorithms is not easy, and some caution is needed when interpreting this result. None-

theless, the median-joining network (Figure S1) clearly supported the notion that forcing the monophyly for *Wolbachia* lineages from *Balloniscus* would result in a scenario which is less consistent with these data.

In conclusion, like other South American arthropods (Shoemaker *et al*., 2000; Ciociola Jr *et al*., 2001; Ono *et al*., 2001; Selivon *et al*., 2002; Vega *et al*., 2002; Dittmar and Whiting, 2004; Heukelbach *et al*., 2004; Rocha *et al*., 2005; Cônsoli and Katajima, 2006; Souza *et al*., 2009), *Wolbachia* also infects South American terrestrial isopods. The population-based approach we took allowed a more accurate estimation of prevalence rates in these species, and permitted the discovery of a high genetic diversity of *Wolbachia* isolates.

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Internet Resources

RDP (Ribosomal Database Project), http://rdp.cme.msu.edu/ (accessed September 15, 2011).

Network version 4.610 software, http://www.fluxus-engineering.com/sharenet.htm (accessed October 10, 2011).

Beast (Bayesian evolutionary analysis sampling trees) version 1.7.4 software, http://evolve.zoo.ox.ac.uk/beast/ (accessed September 10, 2011).

Supplementary Material

The following online material is available for this article:

Figure S1 - Median-joining network of *Wolbachia* 16S rDNA sequences from *Balloniscus* species.

This material is available as part of the online article from http://www.scielo.br/gmb.

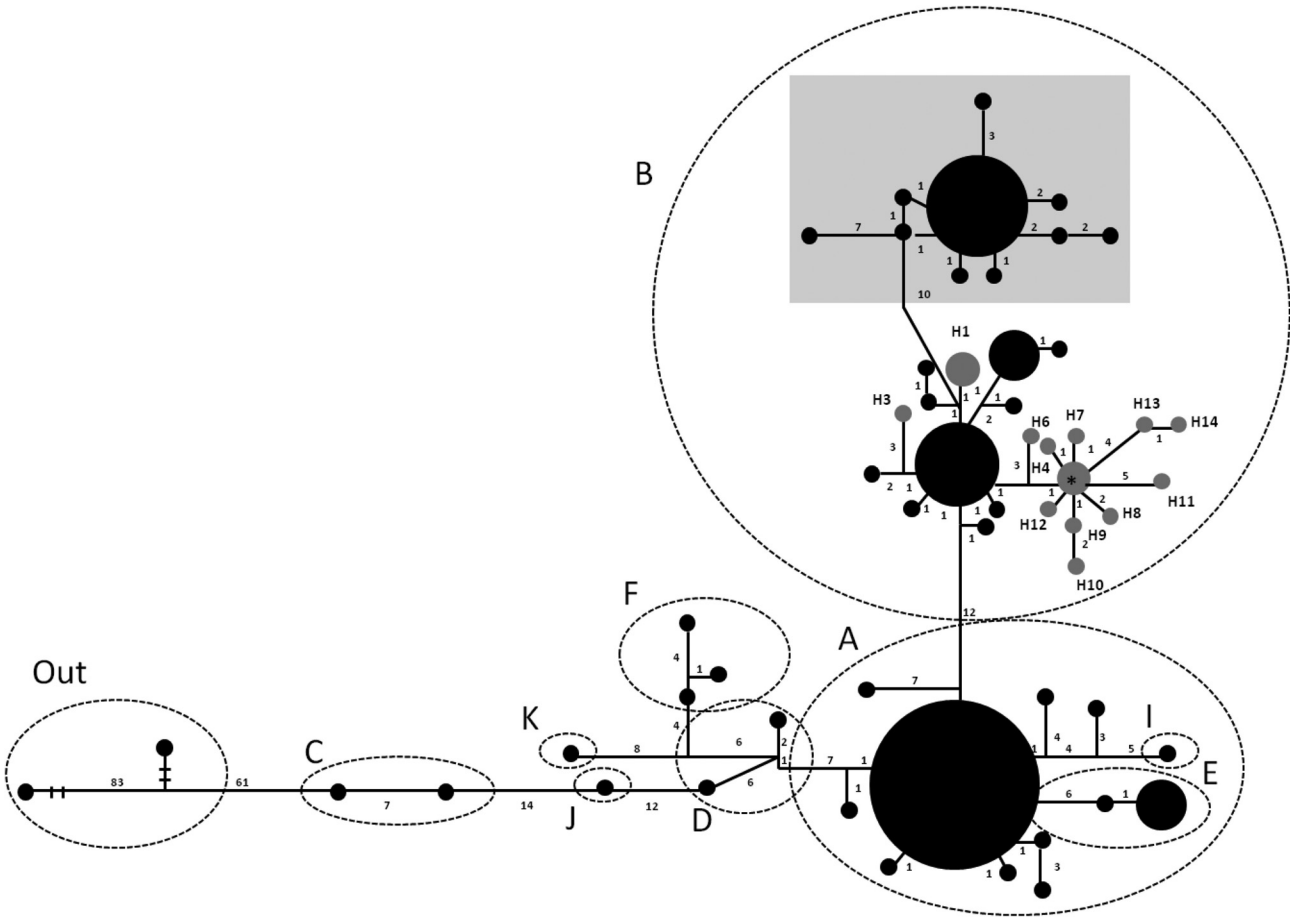

Figure S1 - Median-joining network of *Wolbachia* 16S rDNA sequences from *Balloniscus* species. A, B, C, D, E, F, I and J represent *Wolbachia* supergroups traditionally recognized and Out identifies the sequences used as outgroup (see Figure 2); The *Oni* clade is represented inside a grey square; numbers associated with some branches represent the number of mutational steps; H1, H3, H4, H5, H6, H7, H8, H9, H10, H11, 12, H13 and H14 (*H2) represent the different sequences found in *Balloniscus*, and are represented in grey. *Wolbachia* 16S sequences obtained from other species and used for the phylogenetic analysis (Figure 2) are represented in black. The size of the circles is proportional to the number of sequences considering all individuals.