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PRIMARY RESEARCH PAPER

First detection of the crayfish plague pathogen *Aphanomyces astaci* in South America: a high potential risk to native crayfish

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Abstract The crayfish plague pathogen, *Aphanomyces astaci*, is a fungal-like organism (Oomycetes), specialized in parasitizing freshwater crayfish species. Crayfish plague is a disease that has caused losses of indigenous crayfish populations, especially in Europe. The pathogen chronically infects North American endemic crayfish, such as *Procambarus clarkii*, which is considered an invasive species in several continents, including South America. Using molecular tools, quantitative PCR, and conventional PCR, we detected this pathogen in feral *P. clarkii* populations established in southeastern Brazil. This is an alarming result because in South America, especially in Brazil, there is considerable endemic crayfish species diversity, especially in the genus *Parastacus*. Possible contacts between *P. clarkii* and the endemic crayfish could be seen as a major threat to the native crayfish, mainly because of the possibility of *A. astaci* transmission. Furthermore, our results indicate preliminary evidence of possible *A. astaci* infection, agent level A2, in two sympatric native species, namely *Parastacus defossus* and *Parastacus pilimanus*. In this study, we provide the first overview concerning the presence of the crayfish plague pathogen, *A. astaci*, in South America.

Keywords *Procambarus clarkii* · *Parastacus* · Astacidea · PCR · qPCR · RT-PCR · Alien species · Invasive species · Conservation · Molecular tools

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Introduction

Crayfish plague is a disease caused by the pathogen *Aphanomyces astaci* Schikora (Oomycetes, Saprolegniaceae) (Souty-Grosset et al., 2006). One of the many carriers of *A. astaci* is *Procambarus clarkii* (Girard, 1852) (Astacidea, Cambaridae), originally from North America, and popularly known as the red swamp crayfish (Barbaresi et al., 2007; Hänfling et al., 2011).

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This crayfish may become infected without developing the gross symptoms of the disease or increased mortality (Unestam, 1969; Aquiloni et al., 2011), and, thus, is capable of acting as a chronic carrier and permanent reservoir of the pathogen. In contrast, some other crayfish of North American origin may show increased mortality due to *A. astaci* infection (Thörnqvist & Söderhäll, 1993; Aydin et al., 2014; Jussila et al., 2014a, b, 2015b; Edsman et al., 2015).

High levels of mortality are usually observed within a few months of when *A. astaci* first infects a crayfish population of a susceptible species (Alderman, 1996; Souty-Grosset et al., 2006; Makkonen et al., 2012, 2014; Jussila et al., 2015a). In Europe, mass mortalities caused by crayfish plague epidemics had already started before the introduction of the North American crayfish, as the pathogen *A. astaci* was first introduced into the continent with an unknown host. This host, based on current understanding, is not found in Europe (Grandjean et al., 2014; Kozubíková-Balcarová et al., 2014). A second wave of epidemics was caused by the massive introduction of North American crayfish species from the 1960s onwards, inducing further damage and establishing widespread permanent host populations for *A. astaci* (Unestam, 1972; Alderman, 1996; Söderhäll & Cerenius, 1999; Souty-Grosset et al., 2006; Kozubíková-Balcarová et al., 2014). Currently, one of the main transmission routes of *A. astaci* is the colonization of new habitats by the North American crayfish host species (Holdich et al., 2009; OIE, 2012).

In Brazil, the first reported occurrence of *P. clarkii* was in the State of São Paulo (Huner, 1986), and further reports have been made of several feral *P. clarkii* populations in recent years (Magalhães et al., 2005; Silva & Bueno, 2005; Loureiro et al., 2015). Commercial and illegal trading of crayfish as ornamental species has been suggested as the pathway of introducing *P. clarkii* into Brazil (Loureiro et al., 2015). *Procambarus clarkii* maintained as pets, but released later alive to the environment, may have adapted quickly and successfully, establishing populations in some ponds and lakes (Loureiro et al., 2015). This has happened in spite of the fact that the trade or possession of *P. clarkii* has been prohibited by federal laws since 2008 by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, 2008). Illegal pet trade of *P. clarkii* has been reported not only in the State of São Paulo, but also in other regions inhabited by native freshwater crayfish, such as in Porto Alegre City in south Brazil (Loureiro et al., 2015). The distribution of *P. clarkii* in Brazil follows predictions from dispersal models for this species (Palaoro et al., 2013), and indicates a possible threat to native Brazilian freshwater crayfish in the future.

A total of 13 crayfish species, belonging to three genera (*Parastacus*, *Virilastacus* and *Samastacus*), are native to South America. They are distributed in southern Brazil, Uruguay, northeast and southern Argentina, and central-southern Chile (Buckup, 2003; Rudolph, 2013; Almerão et al., 2014). Brazil hosts South American endemic parastacid crayfish species, which may be infected by *A. astaci* via the introduced *P. clarkii* (Loureiro et al., 2015). The contact among *P. clarkii* (infected with *A. astaci*) with the native crayfish may result in epidemics, thus, negative consequences to diversity, similar to that experienced in Europe (Holdich et al., 2009; Tuffs & Oidtmann, 2011). The threat is heightened by the highly invasive nature of *P. clarkii* (Barbaresi & Gherardi, 2001; Gherardi et al., 2002; Gherardi, 2006), and also by the possibility of human translocations (Magalhães et al., 2005; Silva & Bueno, 2005). *P. clarkii* also seems to be capable of adapting to cooler climatic conditions (as in southern South America), as has been observed in northern Germany (Chucholl, 2011; Veselý et al., 2015; Jörn Panteleit, personal communication), The Netherlands, and southern England (Kouba et al., 2014).

Our aim was to investigate the presence of the crayfish plague pathogen *A. astaci* in Brazilian feral populations of *P. clarkii* using molecular tools [TaqMan MGB quantitative PCR (qPCR) and conventional PCR]. In addition, we investigated the presence of the *A. astaci* in selected native crayfish species, namely *Parastacus brasiliensis* (Von Martens, 1869), *Parastacus pilimanus* (Von Martens, 1869), and *Parastacus defossus* Faxon, 1898, which have not yet been recorded in sympatry with *P. clarkii* populations, to study possible latent *A. astaci* infections in native Brazilian crayfish.

Material and methods

Crayfish sampling

Procambarus clarkii were collected (from April 2012 to February 2013) from different populations established in the State of São Paulo, Brazil, and a sample

was obtained from a pet shop (PS), in Porto Alegre City, State of Rio Grande do Sul, Brazil (Fig. 1; Table 1). The specimens from the wild were captured using hand-nets and traps, and were individually euthanized by freezing, and then stored in 100% ethanol. The same sampling procedures were applied to *P. brasiliensis*, *P. pilimanus*, and *P. defossus*, which were collected in the State of Rio Grande do Sul, Brazil. A total of 160 individuals from 15 different populations or origins were collected.

Crayfish dissection and DNA extraction

The following procedures are based on and modified from Oidtmann et al. (2006), Vrålstad et al. (2009) and Filipová et al. (2013). Individual crayfish were dissected aseptically to remove presumably infected tissues (Oidtmann et al., 2006): abdominal ventral cuticle: 0.5 cm^2, both uropods, and two pereopod coxa. All dissections were conducted at the Laboratório de Carcinologia, Universidade Federal de Rio Grande do Sul, Porto Alegre City, Brazil. All material was preserved in 70% ethanol, and then transported to France for the following procedures at the Laboratoire Ecologie & Biologie des Interactions, Université de Poitiers, Poitiers City, France.

The dissected tissues were aseptically cut by scissors and dried on a heating block (75°C) for about 5 min. Then, 360 µl of ATL buffer (DNeasy tissue kit, Qiagen), and sterilized steel beads (one portion:

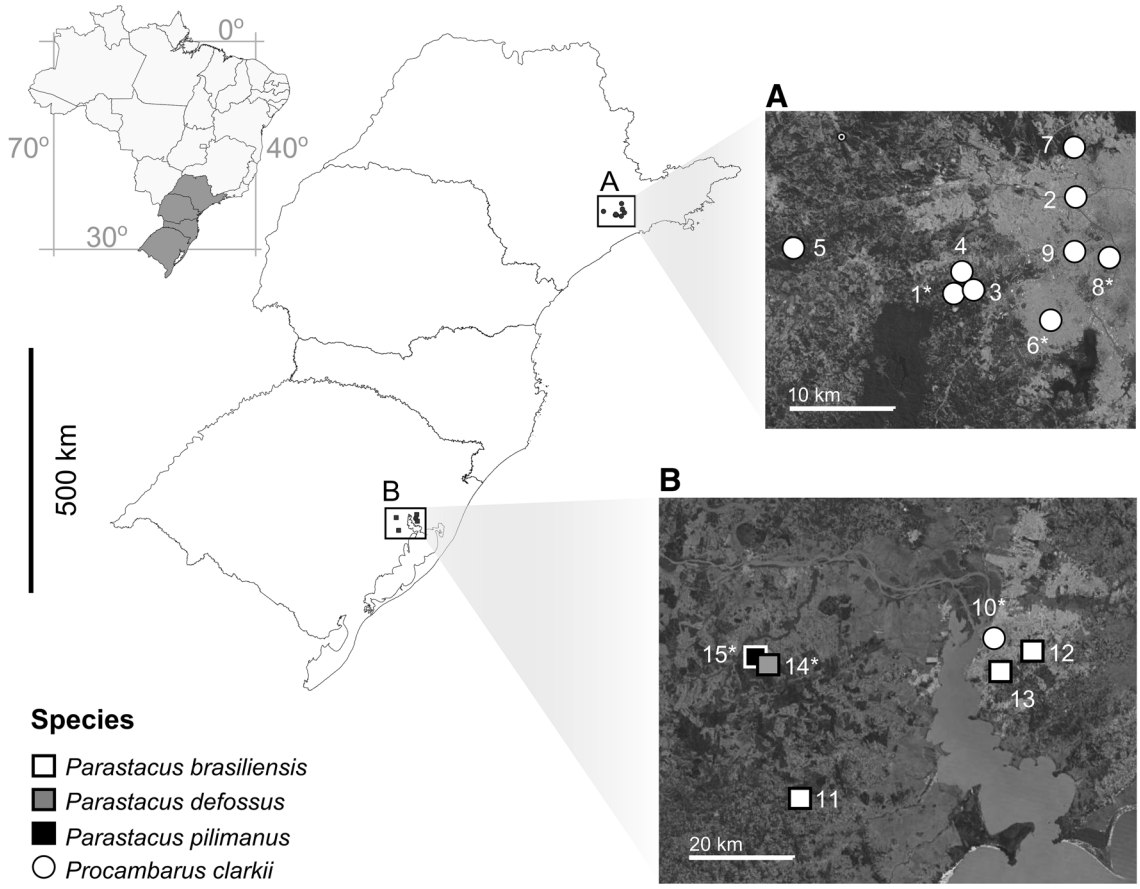

Fig. 1 Brazil and southern states of Brazil. **A** Locations in the State of São Paulo. **B** Locations in State of Rio Grande do Sul. *Population or origins identified *Aphanomyces astaci*-positive (by qPCR or ITS-PCR). *Procambarus clarkii* populations or origins: *1** EM, *2* VRE, *3* APA, *4* CO, *5* SRO, *6** SD, *7* JAR, *8** AV, *9* CEU, and *10** PS. *Parastacus brasiliensis* populations: *11* MP, *12* MS, and *13* ZS. *Parastacus pilimanus* population: *14** EA. *Parastacus defossus* population: *15** EA. Please find the acronyms list of the populations/origins in Table 1

Table 1 Location list of *Procambarus clarkii*, *Parastacus brasiliensis*, *Parastacus pilimanus*, and *Parastacus defossus* collected in Brazil

Population acronyms	Location, city, state	Geographical location	# of analyzed individuals	# of *Aphanomyces astaci*-positive	ITS-PCR	Level of infection: no. individuals
Procambarus clarkii						
1 EM[a]	Pesqueiro do Gaúcho (FP), Embu das Artes, São Paulo	23°38′07″S 46°53′45″W	25	16	1	A2:14, A5:1, A7:1
2 VRE	Vila dos Remédios Park (UL), São Paulo, São Paulo	23°30′56″S 46°44′57″W	1	0	0	–
3 APA	APA Embu-Verde (NA), Embu das Artes, São Paulo	23°38′16″S 46°52′29″W	1	0	0	–
4 CO	João Hara (FP), Cotia, São Paulo	23°37′13″S 46°53′28″W	11	2	0	A5:2
5 SRO	Private area (NA), São Roque, São Paulo	23°35′08″S 47°06′22″W	11	0	0	–
6 SD[a]	Santo Dias Park (UL), São Paulo, São Paulo	23°39′53″S 46°46′23″W	11	0	1	–
7 JAR	Jaraguá Aldeia Indígena (CA), São Paulo, São Paulo	23°27′49″S 46°45′17″W	2	1	0	A5:1
8 AV[a]	Alfredo Volpi Park (UL), São Paulo, São Paulo	23°35′16″S 46°42′09″W	36	6	1	A2:3, A5:2, A7:1
9 CEU	CEU Butantã (UL), São Paulo, São Paulo	23°34′51″S 46°45′03″W	4	0	0	–
10 PS[a]	Pet Shop, Porto Alegre, Rio Grande do Sul	–	13	3	2	A2:3
Parastacus brasiliensis						
11 MP	Morro Cerro Negro (NA), Mariana Pimentel, Rio Grande do Sul	30°20′44″S 51°34′08″W	15	0	0	–
12 MS	Campus do Vale UFRGS (NA), Morro Santana, Rio Grande do Sul	30°03′46″S 51°07′26″W	1	0	0	–
13 ZS	Zona Sul (NA), Porto Alegre, Rio Grande do Sul	30° 05′ 52″S 51° 11′ 30″W	10	0	0	–
Parastacus pilimanus						
14 EA	Estação Agronômica UFRGS (NA), Eldorado do Sul, Rio Grande do Sul	30°20′44″S 51°34′08″W	7	1	0	A2:1
Parastacus defossus						
15 EA	Estação Agronômica UFRGS (NA), Eldorado do Sul, Rio Grande do Sul	30°20′44″S 51°34′08″W	12	2	0	A2:2
	Total		160	31	5	–

Aphanomyces astaci-positive: number of positives by qPCR

ITS-PCR number of positives by ITS-PCR analysis, *Level of infection* population agent level of infection obtained by TaqMan qPCR, *FP* fishing pond, *UL* urban lakes, *NA* natural area, *CA* conservation area

[a] Populations or origins identified *A. astaci*-positive by ITS-PCR

0.10 g) were added to each sample; and the tissues were then mechanically ground in crusher equipment (Bullet Blender 24) for 10 min at maximum velocity. After grinding, the beads were removed and the DNA was extracted according to the manufacturer's protocol. The quantities of the ATL buffer (360 µl) and proteinase K (40 µl) were different to those suggested in the protocol. An additional centrifugation step was introduced after the digestion step. Only the fluid supernatant after digestion was transferred into the columns, since partially undigested cuticle particles would otherwise have blocked these. The DNA quantity and quality were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Samples with low quantity and quality of DNA were reprocessed starting from the first step. We adjusted the DNA template concentration after isolation to standard values based on NanoDrop readings (we adjusted the isolate volume).

Preparation of standards for qPCR

The standards (calibration curve), based on a four-fold dilution series of genomic DNA, were made using a DNA extraction from a pure mycelium of *A. astaci* (UEF8866-2) from the Department on Environmental and Biological Sciences, University of Eastern Finland, Kuopio Campus, according to procedures described by Vrålstad et al. (2009), using a real-time thermal cycler LightCycler 480 II (Roche). The calibration of the standard curve was made according to Vrålstad et al. (2009). The quantity of the pathogen DNA in these standards, expressed in PCR forming units (PFUs), was 4^{-2} equals 3,758,096 PFU, 4^{-4} equals 251,658 PFU, 4^{-6} equals 14,680 PFU, 4^{-8} equals 918 PFU, and 4^{-10} equals 57 PFU.

Real-Time qPCR

The procedures were based on and modified from Vrålstad et al. (2009) and Filipová et al. (2013). The qPCR analysis was conducted with primers and probe published by Vrålstad et al. (2009), targeting a 59 bp fragment in the internal transcribed spacer 1 regions of *A. astaci*. The reaction setup and qPCR conditions followed Vrålstad et al. (2009), except that the LightCycler 480 II qPCR machine was used. To test the impact of inhibition that might influence the detection efficiency, each sample template was analyzed using 20 and 2 ng/ml DNA (10-fold diluted replicate) (Vrålstad et al., 2009; Filipová et al., 2013). When some effects of inhibition were occasionally detected (mostly in samples with a low agent level), the PFU values were estimated as described in Kozubíková et al. (2011) and Filipová et al. (2013).

qPCR analysis

The samples were classified into semi-quantitative categories of pathogen load based on PFU values (Vrålstad et al., 2009). The categories ranged from A0 (negative: no traces of *A. astaci* DNA) to A7 (extremely high amounts of *A. astaci* DNA in the sample). As proposed by Vrålstad et al. (2009), only individuals with agent level A2 and higher are considered infected. The interpretation for the agent level A1 indicates insufficient traces of *A. astaci* DNA for the diagnosis (corresponding to <5 PFU). It may also indicate a false positive or minor contamination during the analysis; therefore, A1 is not considered as a positive result (Vrålstad et al., 2009; Kozubíková et al., 2011; Filipová et al., 2013).

ITS-PCR preparation and analysis

The procedures below were modified from Oidtmann et al. (2006). The ITS 1 and 2 spacer DNA regions and the gene for 5.8S rRNA in between them were amplified by PCR (with primers: 42 and 640) in an iCycler thermal cycler (BioRad). Primers 42 and 640 are specific to *A. astaci* (Oidtmann et al., 2006), although some very closely related species may cause false positive results (Kozubíková et al., 2009). The amplifications were carried out in a 25 µl reaction volume. The mix contained 5 µl Taq buffer, 2.5 µl dNTPs mixture, and 10 µM µl from each primer: 42 and 640 (Oidtmann et al., 2004, 2006) at a final concentration of 0.125 µl of GoTaq PCR enzyme, 0.5 or 1.0 µl of template DNA, and nuclease free water H_2O to complete the reaction volume. Thermal cycles were as follows: 95°C for 3 min, ×40 (30 s at 95°C, 30 s at 62°C, and 1 min at 72°C), followed by 5 min at 72°C. Amplicons with an expected length of 569 bp were obtained. To ensure that the lack of a product was not due to insufficient amounts of DNA being submitted to the PCR, a positive control was run. The positive control was the *A. astaci* DNA extracted from a mycelium (isolate UEF8866-2) diluted at 2 ng/

µl of DNA concentration. A negative control was also run—the same mix concentrations with no DNA. The amplified DNA was analyzed/detected by electrophoresis in agarose gel (1.5%) using 5 µl of the PCR reaction product. Afterwards, the gel was submerged in a solution containing 0.2 µg ml ethidium bromide for contrast photography.

Results

Results from the real-time qPCR

We detected the presence of the crayfish plague pathogen in five out of the nine feral *P. clarkii* populations studied in the State of São Paulo (EM, CO, SD, JAR and AV) (Table 1). In total, 25 out of 102 crayfish were *A. astaci*-positive. The agent level of infection in these individuals ranged from A2 to A7 (Table 1). The presence of the pathogen was also detected in 3 out of 13 analyzed individuals from the PS in Porto Alegre City, State of Rio Grande do Sul (PS), all with an agent level of infection at A2.

We did not detect the presence of *A. astaci* in the analyzed *P. brasiliensis* from natural populations. An intriguing result was the presence of *A. astaci* DNA in 1 out of 7 individuals of *P. pilimanus* and 2 out of 12 individuals of *P. defossus* from the native sympatric populations from Eldorado do Sul (EA) (about 30 km from Porto Alegre City). All three positive samples had the agent level of infection at A2 (Table 1).

Results from the ITS-PCR

Using the conventional PCR method for ITS regions (primers: 42 and 640), crayfish plague was detected in three out of nine *P. clarkii* populations established in the State of São Paulo (EM, SD and AV). The number of *A. astaci*-positive detected by ITS-PCR was 1 out of 25 individuals from EM, 1 out of 11 individuals from SD, and 1 out of 36 individuals from AV. We also detected the presence of the pathogen in 2 out of 13 individuals obtained from the PS in Porto Alegre City.

Discussion

We present here the first detection of the crayfish plague pathogen *A. astaci* in South America, in the alien freshwater crayfish (*P. clarkii*), and also present preliminary evidence of possible infection in native crayfish in Brazil. We positively detected *A. astaci* in five feral *P. clarkii* populations in the State of São Paulo. We also detected this pathogen in *P. clarkii* from a PS in the State of Rio Grande do Sul, even though the agent level of infection was quite low in most of the studied individuals (A2). A similar A2 level is observed in various studies in Europe cited in the current study (also in Schrimpf et al., 2012). The results are alarming as the chronic *A. astaci* carriers have been reported to release *A. astaci* spores continuously (Strand et al., 2012; Svoboda et al., 2013). High-agent levels A5 and A7 were also reported in the current study (Table 1).

There is now a verified threat of this pathogen in Brazil carried in this case by the alien crayfish, *P. clarkii*. Thus, the *A. astaci* could be potentially spreading to natural populations of endemic Brazilian crayfish, and possibly also to other decapods (Schrimpf et al., 2014; Svoboda et al., 2014a, b). Since *A. astaci* epidemics in Europe resulted in eradication of most wild stocks of crayfish and threats to remaining stocks by different *A. astaci* genotypes (Alderman, 1996; Souty-Grosset et al., 2006; Jussila et al., 2015a), there is great reason for concern over *A. astaci* spread in South America. *Aphanomyces astaci* epidemics are considered one of the worst causes of crayfish population declines or eradications (Edgerton et al., 2004; Souty-Grosset et al., 2006; Holdich et al., 2009). However, there are, so far, no reports of serious negative impacts from *A. astaci* presence outside of Europe. In addition to the threat posed by the pathogen, there is also a threat from the competition with the invasive alien carrier crayfish species (Holdich et al., 2009). The general consequences of contact among *P. clarkii* and native Brazilian crayfish species could cause profound changes in native crustacean diversity (Alderman, 1996; Kozubíková et al., 2009, 2010). Others have shown that *A. astaci* can infect decapods other than crayfish, such as crabs, and probably, also shrimps (Schrimpf et al., 2014; Svoboda et al., 2014a, b). The level of extinction risk differs among crayfish families, with proportionally more threatened species in the Parastacidae and Astacidea than in the Cambaridae (Richman et al., 2015), while actual studies on the South American species are lacking.

The *A. astaci* detection techniques used in this study (qPCR and ITS-PCR) yielded positive results.

We did not have specific objectives to compare these methods, but we found significant differences in the numbers of *A. astaci*-positive samples between them. Kozubíková et al. (2011) showed that the results based on the conventional PCR were not substantially influenced by false positives, but might have suffered from some false negatives at low agent levels. Using pure mycelium and zoospores, Tuffs & Oidtmann (2011) compared the two methods used in the current study. They considered both methods to be time and resource effective, but clearly demonstrated that the qPCR is more sensitive than ITS-PCR. Combining alternative methods may, therefore, provide more reliable conclusions about the pathogen's presence. In our study, the TaqMan MGB-probe qPCR detected 31 infected individuals, while the conventional ITS-PCR detected only five individuals' *A. astaci*-positive. Almost all the individuals detected as positive for *A. astaci* using the ITS-PCR technique were also detected *A. astaci*-positive using the qPCR technique. One exception was an individual from the SD population, showing amplification in ITS-PCR, while the qPCR remained negative. This single result can be a possible false positive detection, caused by some closely related species of *A. astaci*, as well as contamination of the sample during its handling and laboratory work. However, the latter option is considered rather unlikely, as best laboratory practices were exercised while preparing the sample. Furthermore, all negative controls included for the sample preparation and PCR also remained negative, verifying proper laboratory protocol.

Even though there are no reports of *P. clarkii* living in sympatry with the native Brazilian crayfish species, they are already causing damage to the local ecosystems as competitors, predators, and habitat modifiers (Loureiro et al., 2015). The potential encounter of *P. clarkii* with the native Brazilian crayfish may cause negative consequences for the biodiversity, similar to what has happened in Europe (Barbaresi et al., 2007; Holdich et al., 2009; Cammà et al., 2010). The native Brazilian freshwater crayfish species have restricted distributions (endemic) (Almerão et al., 2014), and, thus, are vulnerable to instabilities in their habitats.

We detected *A. astaci*-positive *P. clarkii* individuals from a PS in Porto Alegre City, which is within the known distribution area of the endemic crayfish species (Buckup, 2003; Almerão et al., 2014). Other studies have also shown the crayfish pet trade as a means of introducing disease agents, including *A. astaci*, into European aquatic ecosystems (Longshaw et al., 2012; Keller et al., 2014; Mrugała et al., 2015). The potential of *P. clarkii* to establish invasive populations is well known (Barbaresi et al., 2007; Jones et al., 2009), and illegal trading of alien pet crayfish, thus, poses a threat to the native Brazilian freshwater species and may help to spread *A. astaci* further. This threat is real, despite the fact that Brazilian federal laws are prohibiting importation, selling, and possession of *P. clarkii* and other alien crayfish (IBAMA, 2008; Magalhães & Andrade, 2015). Furthermore, it was recently shown that *P. clarkii* has the potential to disperse also to those Brazilian natural ecosystems that are currently occupied by the native freshwater crayfish *Parastacus* spp. (Palaoro et al., 2013). However, the distribution ranges of the native Brazilian freshwater crayfish are not entirely known, and further studies are needed to fully understand the native crayfish ecology (Almerão et al., 2014; Loureiro et al., 2015). In addition to the efforts of Loureiro et al. (2015) on tracking and mapping distribution of *P. clarkii*, increased sampling efforts are required to identify possible new settlements of this alien freshwater crayfish in Brazil.

The qPCR analyses also showed signs of *A. astaci* infection in native crayfish species, namely *P. pilimanus* and *P. defossus* from Eldorado do Sul, where these two species are living in sympatry. However, these results should be observed with caution, as there is no way to confirm the results of the A2 agent level by sequencing or by any other method, as the amount of the pathogen DNA in tissue was too low to obtain amplification in the conventional PCR. Therefore, further sampling and analyses of these populations are needed to confirm the results. Due to the serious nature of the *A. astaci* infection, even for cases that do not apparently seem to be alarming, care must be taken, because this disease should not be given any opportunity to spread to pristine freshwater crayfish stocks. Bearing that in mind, several reports have been published about native freshwater crayfish from Europe being latently infected with *A. astaci* (Jussila et al., 2011; Viljamaa-Dirks et al., 2011; Kokko et al., 2012; Svoboda et al., 2012; Kušar et al., 2013). How the studied Brazilian native crayfish stocks became infected by *A. astaci* remains unanswered, as there is currently no evidence that they have been in contact with feral populations of *P. clarkii*. Other transmission

routes could be moving wet equipment, clothes, fishing gear, fish, or even water from infected areas (Reynolds & Souty-Grosset, 2012).

The main contributions of this study are the first detection of *A. astaci* in South America in alien crayfish and preliminary evidence of the presence of *A. astaci* in native crayfish in Brazil. This finding could have serious consequences, as native freshwater crayfish could now be under threat of mass mortality due to *A. astaci* epidemics. This opens up opportunities for research, as there is a need to understand the infection pathways into and within Brazil, and to reveal the possible variety of *A. astaci* genotypes infecting different freshwater crayfish species in this geographic region.

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