

## Research Article

## Investigation of ornamental crayfish reveals new carrier species of the crayfish plague pathogen (*Aphanomyces astaci*)

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### Abstract

Several North American crayfish species have so far been identified as carriers of the crayfish plague agent *Aphanomyces astaci*. The pathogen is responsible for the declines of thousands of European crayfish populations. Currently, one of the introduction pathways of North American crayfish species is the aquarium trade which may sometimes be followed by intentional release or unintentional escape of the pet species into the wild. We investigated 85 samples of North American and New Guinean species, available through the aquarium trade, for their possible infection with *A. astaci*. Crayfish plague infection was examined by applying real-time PCR. Besides morphological identification, we sequenced a fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene for crayfish species confirmation. Additionally, sequence analysis of nuclear DNA was conducted to identify the *A. astaci* lineage of moderate to highly infected crayfish. A total of 11 of the 85 analyzed crayfish individuals tested positive for an *A. astaci* infection, of which nine species are for the first time identified as carriers of *A. astaci*. No new genetic lineages of *A. astaci* were identified. The results confirm that, due to the positive carrier status of tested crayfish, the aquarium trade in Europe can facilitate the spread of *A. astaci* and can thus be a significant threat to the indigenous crayfish and the environment.

**Key words:** freshwater crayfish, invasive species, aquarium trade, real-time PCR, COI sequence analysis

### Introduction

Translocations and range expansions of non-indigenous species often disturb the ecological balance of natural communities and native ecosystems by increased predation, competition and habitat alteration (Reynolds 2011). In the past, non-indigenous crayfish species (NICS) have intentionally been introduced and stocked into European waters to compensate for the native crayfish populations that were in decline due to mass mortalities caused by *Aphanomyces astaci* Schikora, 1906 infections, also known as crayfish plague epidemics (Alderman 1996). Old NICS (i.e., NICS introduced before 1975), the signal crayfish *Pacifastacus leniusculus* (Dana, 1852), the spiny-cheek crayfish *Orconectes limosus* (Rafinesque, 1817) and the red swamp crayfish *Procambarus clarkii* (Girard, 1852), have

long been established in European waters (Holdich et al. 2009), and today occur in many European countries in higher densities than indigenous crayfish species (ICS). This replacement can mainly be attributed to competition and crayfish plague transmission (Reynolds 2011), leading to loss of habitat and death for ICS (Westman et al. 2002; Holdich et al. 2009). Crayfish plague disease is probably the most important factor in the population declines of ICS. The oomycete *A. astaci* is a parasite which can be transmitted via infected crayfish (reviewed by Longshaw 2011), but also via infected crab species, in particular *Eriocheir sinensis* H. Milne Edwards, 1853 (Schrimpf et al. 2014; Svoboda et al. 2014). Wild fish and fish stocking, as well as the transport of fishing gear and traps, may be responsible for the further spread of *A. astaci* (Alderman 1996; Oidtmann et al. 2002).

In contrast to the Old NICS, there are the New NICS (i.e., NICS introduced after 1980) which were introduced mainly through the aquarium trade and for aquaculture purposes (Holdich et al. 2009). Although the animal trade is known to provide opportunities for biological invasions, crayfish have only recently experienced increased popularity as exotic pets (Holdich et al. 2009; Chucholl 2013; Patoka et al. 2014). As a result, releases and escapes from aquaria and aquaculture are currently among the main pathways for invasions of New NICS into Central Europe (Alderman 1996; Holdich et al. 2009; Peay 2009). The main factors which increase the probability for releases into nature from aquaria are large body size and high availability in the aquarium trade (Chucholl 2013). The threat arising from the crayfish aquarium trade is high, particularly in Germany where at least 120 alien crayfish species can be purchased. About seven new crayfish species per year were imported for the aquarium trade between 2005 and 2009 (Chucholl 2013).

Since its first discovery in Europe in the late 19<sup>th</sup> century, *A. astaci* seems to have been introduced into Europe numerous times, resulting in the introduction of different lineages of *A. astaci* from different locations in North America (Huang et al. 1994; Diéguez-Urbeondo et al. 1995; Kozubíková et al. 2011; Viljamaa-Dirks et al. 2013). So far, five lineages of *A. astaci* have been identified. Signal crayfish (*P. leniusculus*) of American and Canadian origin have been shown to carry the lineages PsI or PsII, respectively. American red swamp crayfish (*P. clarkii*) carry the lineage Pc and spiny-cheek crayfish (*O. limosus*) the lineage Or (Huang et al. 1994; Diéguez-Urbeondo et al. 1995; Rezinciuc et al. 2013; Kozubíková et al. 2011). Lineage As was first isolated from European noble crayfish *Astacus astacus* (Linnaeus, 1758) while its original American host species remains unknown (Huang et al. 1994; Makkonen et al. 2012a; Viljamaa-Dirks et al. 2013). Following up on the allocation of these different lineages into different genetic groups as first done by Huang et al. (1994), Rezinciuc et al. (2013) revealed through AFLP-PCR that there is some genetic variation within these different genetic lineages of *A. astaci*. This was further confirmed through the development of microsatellite markers, showing differences in allele sizes within genetic lineages of *A. astaci* (Grandjean et al. 2014; Maguire et al. 2016). Thus, the genetic variation of *A. astaci* is probably higher than revealed by RAPD analysis (Huang et al. 1994). It is therefore reasonable to assume that different genetic lineages of *A. astaci* each consist of numerous different genotypes.

While native European crayfish, i.e. the stone crayfish (*Austropotamobius torrentium* Schrank, 1803) and the IUCN Red-List Vulnerable noble crayfish (*A. astacus*) (IUCN 2015), are highly susceptible to *A. astaci* infections and as a consequence can undergo mass population declines (Alderman 1996; Kozubíková et al. 2011; Filipová et al. 2013), North American crayfish can usually resist an *A. astaci* infection due to their coevolved immune system, unless they are exposed to additional stress (Söderhäll and Cerenius 1992; Alderman 1996; Cerenius et al. 2003; Aydin et al. 2014). However, a growing number of studies have found chronic infections in populations of noble crayfish (Makkonen et al. 2012b) stone crayfish (Kušar et al. 2013) or white-clawed crayfish (*Austropotamobius pallipes* Lereboullet, 1858) (Maguire et al. 2016), which suggests that they might have developed an increased resistance to *A. astaci* infection.

Of the 120 crayfish species available in the German aquarium trade, 105 have been considered as potential *A. astaci* vectors because of their North or Central American origin (Chucholl 2013). By 2014, six NICS had been identified as carriers of *A. astaci*: *Pacifastacus leniusculus* (Unestam and Weiss 1970), *O. limosus* (Vey et al. 1983), *P. clarkii* (Diéguez-Urbeondo and Söderhäll 1993), *Orconectes immunis* (Hagen, 1870) (Schrimpf et al. 2013), *Procambarus fallax* f. *virginalis* Martin et al., 2010 (Keller et al. 2014) and *Orconectes virilis* (Hagen, 1860) (Tilmans et al. 2014). Mrugała et al. (2015) recently identified seven crayfish species from the aquarium trade as new potential carriers of *A. astaci*, six of which originate from North America and one from Australia. However, as these potential carriers were not confirmed by isolation of *A. astaci*, these results should be interpreted with caution. They also showed that frequent misidentification of crayfish species occurs which is why crayfish might sometimes be sold with the wrong species names.

Complementary to Mrugała et al. (2015), in this study we investigated 85 crayfish individuals from 50 morphologically identified species, of mostly North American or Central American origin (USA, Canada, Mexico, Guatemala) for possible *A. astaci* infection. Many of the studied species have never before been tested for an infection with *A. astaci*. All studied crayfish species can be bought in the German aquarium trade and are thus a potential threat to native ecosystems if released into the wild. Additionally, two of the studied species originate from Papua and West New Guinea, which allows for the testing of a possible horizontal transfer of *A. astaci* in the aquarium trade (Mrugała et al. 2015), as species

from Australasia can be assumed to be *A. astaci*-free in their natural environment (Unestam 1976).

## Material and methods

### *Crayfish samples and species identification*

The 85 individual crayfish from 50 different ornamental species, based on morphological identification, were obtained from a German hobby breeder. All studied species belonged to seven genera (*Barbicambarus*, *Cambarus*, *Cherax*, *Fallicambarus*, *Orconectes*, *Pacifastacus* and *Procambarus*). Thirteen of the crayfish individuals could not be identified by the hobby breeder, however he assumed they were 10 different species, based on morphological characterization. After their death they were stored in 70% ethanol, for one month to five years. Individuals from the same species were stored in the same containers. For additional verification of crayfish species identification a fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was sequenced. DNA was extracted from muscle tissue using a standardized protocol ("High Salt DNA Extraction Protocol for removable samples"; Aljanabi and Martinez 1997). The reaction mixture contained 5× PCR buffer, 0.03125 u TaqMan® Taq (all Promega, Mannheim, Germany), 1.5 mM MgCl<sub>2</sub>, 0.5 mM dNTP mix (all Fermentas, St. Leon-Rot, Germany), 0.4 μM of primers LCO1490 and HCO2198 (Folmer et al. 1994) and 2 μL DNA template for a final volume of 20 μL. For the samples that created no results a second attempt was started containing 4 μL DNA and 16 μL master mix. PCR was performed using a Primus 96 Plus Thermal Cycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany) under the following conditions: 4 min 94 °C, 35 × (45 s 94 °C, 45 s 47 °C, 60 s 72 °C) and 10 min 72 °C. PCR products were sequenced on a 3730 DNA Analyzer capillary sequencer (Applied Biosystems, MA, USA) by the company SEQ.IT (Kaiserslautern, Germany). The sequences were edited with the program Geneious R7 (Drummond et al. 2011) and compared with reference sequences from the NCBI GenBank via the Basic Local Alignment Search Tool (BLAST). The truncated alignment of the sequences was 605 base pairs (bp) long. The reference sequence was the sequence with the highest match to the sampled sequence, but at least 95%. We only assumed a species to be confirmed, if the hobby breeder named the same species and if there were no other sequences in GenBank with the same percentage coverage. Otherwise the samples were only assigned to the respective genus without providing a species name.

### *Aphanomyces astaci infection status analysis*

The soft abdominal cuticle, the inner joints of two walking legs and parts of the uropods were cut off for DNA extraction using a CTAB-method (Vrålstad et al. 2009). To assess the *A. astaci* infection status of the exotic crayfish, an ITS region-targeting TaqMan® minor groove binder (MGB) qPCR was conducted after Vrålstad et al. (2009) with some modifications (Schrimpf et al. 2013). Based on the number of PCR forming units (PFU) infection status and agent levels from *A. astaci* specific qPCR were defined according to Vrålstad et al. (2009), where samples with agent level A0 (0 PFU) and A1 (PFU<sub>obs</sub> < 5 PFU) are considered uninfected and agent level A2 (5 PFU ≤ PFU<sub>obs</sub> < 50 PFU) and higher (A3: 50 PFU ≤ PFU<sub>obs</sub> < 10<sup>3</sup> PFU; A4: 10<sup>3</sup> PFU ≤ PFU<sub>obs</sub> < 10<sup>4</sup> PFU; A5: 10<sup>4</sup> PFU ≤ PFU<sub>obs</sub> < 10<sup>5</sup> PFU; A6: 10<sup>5</sup> PFU ≤ PFU<sub>obs</sub> < 10<sup>6</sup> PFU; A7: 10<sup>6</sup> PFU ≤ PFU<sub>obs</sub>) are considered infected with *A. astaci*.

### *Aphanomyces astaci lineage identification*

For *A. astaci* lineage identification we amplified a 370 bp long fragment of the chitinase gene following Makkonen et al. (2012a). The sequence from the chitinase gene only allows determination of lineages As and Pc. Lineages PsI, PsII and Or have the same chitinase sequence and thus cannot be distinguished from one another. The amplification was checked on a 1.5% agarose gel containing 0.5 μg ml<sup>-1</sup> ethidium bromide and the amplified PCR products were then purified with QiaQuick PCR Purification Kit (Qiagen, Germany). The purified PCR products were premixed with AACHiF-primer (Makkonen et al. 2012a) and sent for sequencing to GATC Biotech (Cologne, Germany). The sequences were edited with the program Geneious R7 and the lineage was determined by comparison to reference sequences from GenBank.

## Results

### *Crayfish species identification*

The COI sequence analysis was successful for 55 of the 85 crayfish (Supplementary material Table S1; Genbank accession numbers: KU527854–KU527891). For those individuals the species identification from the hobby breeder is given as well as the closest matching GenBank entry. The alignment was truncated to include good sequences at the 3' as well as the 5' end. For 24 genetically identified specimens no reference sequence was available in GenBank. Of the 31 individuals for which a reference sequence was available and the COI sequencing was successful, 27 (87.1 %) matched the morphological identification

of the hobby breeder, but in four cases (12.9 %) the genetic species determination deviated from the morphological determination. These were the morphological identified specimens *Cambarus striatus* Hay, 1902, *Orconectes hylas* (Faxon, 1890), *Orconectes eupunctus* Williams, 1952 and *Cherax holthuisi* Lukhaup and Pekny, 2006, which had a better genetic identity with the species *Cambarus halli* Hobbs, 1968, *Orconectes quadruncus* Creaser, 1933, *Orconectes obscurus* (Hagen, 1870) and a different *Cherax* sp., respectively.

#### Aphanomyces astaci infection status analysis

DNA of *A. astaci* was detected in 11 out of the 85 samples (12.9 %). Each of the 11 individuals belonged to a different species, and all originated from North or Central America (Table 1). Seven crayfish had low agent levels (A2), while two *Orconectes* species (morphological identification: *Orconectes eupunctus* and *Orconectes hylas*) had moderate agent levels (A3 and A4). *Procambarus llamasii* Villalobos, 1954, originating from Mexico, had a very high agent level (A6). The two specimens from Papua New Guinea or West Guinea, *C. boesemani* and *C. holthuisi*, tested negative for *A. astaci* infection.

#### Aphanomyces astaci lineage identification

The sequencing of the chitinase gene was successful for only two infected crayfish species. *Procambarus llamasii* was a carrier of the Pc-lineage. The *A. astaci* lineage carried by *O. hylas* could be narrowed down to either the Or-, Ps-lineage or a different, as yet unknown, genetic lineage which has the same chitinase sequence.

## Discussion

In this study we tested the *A. astaci* infection status of different crayfish species which are sold in the German aquarium trade. Eleven individuals, each belonging to a different species, tested positive for *A. astaci* (Table 1). Nine species are for the first time identified as carriers of *A. astaci*: three *Orconectes* species (*O. ozarkae* Williams, 1952, *O. neglectus* (Faxon, 1885) and *O. luteus* (Creaser, 1933)), two *Cambarus* species (*C. fasciatus* Hobbs, 1981 and *C. manningi* Hobbs, 1981) and *Procambarus simulans* (Faxon, 1884). For three infected species a genetic confirmation of the morphological species determination was not possible due to reduced sequence similarity compared to the reference sequence obtained from GenBank (*Orconectes eupunctus* and *O. hylas*) or due to lacking COI reference in GenBank (*Procambarus pygmaeus* Hobbs, 1942). Finally, two

**Table 1.** Number of samples for specific agent level of infected crayfish species. The level of infection ranges from A0 (not infected) to A7 (very high level of infection). Positive-tested samples are presented in bold letters.

Species (morphologically determined) (n)	Agent level					
	A0	A1	A2	A3	A4	A6
<i>Cambarus fasciatus</i> (3)	1	1	<b>1</b>			
<i>Cambarus manningi</i> (4)	1	2	<b>1</b>			
<i>Orconectes</i> sp. (1)				<b>1</b>		
<i>Orconectes</i> sp. (1)					<b>1</b>	
<i>Orconectes luteus</i> (1)			<b>1</b>			
<i>Orconectes neglectus</i> (1)			<b>1</b>			
<i>Orconectes ozarkae</i> (1)			<b>1</b>			
<i>Pacifastacus leniusculus</i> (4)	2	1	<b>1</b>			
<i>Procambarus llamasii</i> (1)						<b>1</b>
<i>Procambarus</i> sp. (1)			<b>1</b>			
<i>Procambarus simulans</i> (2)		1	<b>1</b>			
Summary of all specimens	4	5	<b>8</b>	<b>1</b>	<b>1</b>	<b>1</b>

species tested positive in our study were already known to be carriers of *A. astaci* (*P. leniusculus*, Unestam and Weiss 1970; *P. llamasii*, Mrugała et al. 2015). As we received the samples already stored in ethanol, it was not possible to attempt an isolation of *A. astaci* into laboratory culture. This would have been the ultimate proof of an infection of the crayfish with *A. astaci*. Detection of *A. astaci* DNA in this study might, in principle, be due to spore attachment on the cuticles of the crayfish.

Thirteen crayfish samples could not be identified morphologically. This represents a general problem with the crayfish aquarium trade, as crayfish are often misidentified and sold with the wrong name (Mrugała et al. 2015). Although some species might have been misidentified by the hobby breeder, 27 of the morphologically identified individuals showed a good match (sequence similarity > 95%) with the COI reference sequences from GenBank, if a reference was available. Previously published sequences of the genus *Orconectes* (Taylor and Hardman 2002; Taylor and Knouft 2006) did not always confirm the morphological identification by the hobby breeder, highlighting the difficulty of morphological identification especially for North American species. Additionally, a 5% divergence may also include some cryptic species in cambarid crayfish (Mathews et al. 2008; Filipová et al. 2010). In general, a greater effort to create a reliable genetic database for crayfish species identification is necessary, as for ten species from this study no COI reference sequence was available, which may lead to the misidentification of the sample (Filipová et al. 2011).

Unfortunately, for 30 samples the COI sequence analysis was unsuccessful. This may be due to a low DNA quality as a result of suboptimal storing conditions. DNA degradation can also lead to an underestimation of the agent level during screening for pathogen presence. Another reason for COI sequencing failure could be mutations in the primer binding sites of the standard Folmer primers.

Our study comprises small sample sizes, often only one specimen per species and always less than five (Table S1), which makes a negative infection result difficult to interpret. Underestimation of the real number of infected individuals, caused by DNA degradation or the presence of inhibitors, cannot be ruled out. However, those specimens which tested positive for *A. astaci* can be considered as *A. astaci* carrying species, with maybe even a high prevalence of carriers in the sampled stock. For negatively tested specimens, we can only draw conditional conclusions, as the small sample sizes result in a rather large possibility of missing a low prevalence *A. astaci* infection in the main stock itself. Mrugała et al. (2015) showed that *Procambarus enoplosternum* can be carrier of *A. astaci* but the one individual of this species in our study tested negative. Further studies regarding the *A. astaci* infection rate of the other negative-tested species are thus needed.

There are several possible scenarios for how the studied ornamental crayfish got infected with *A. astaci*, either in their native environment before capture or during holding in pet shop tanks. Horizontal transmission of the pathogen between the crayfish is one possibility, since specimens were kept in adjacent aquaria by the crayfish breeder. Contaminated equipment could also have caused horizontal transmission of *A. astaci* between crayfish. As the sequence analysis of the chitinase gene only allows for the discrimination of the *A. astaci* lineages As and Pc, while the remaining lineages Or, PsI and PsII are identical, we were only able to detect the Pc lineage in *P. llamasii*. However, one *Orconectes* species (morphologically identified as *O. hylas*) was either infected with the Or lineage or one of the Ps lineages. It might also be possible however that it was infected with an unknown genetic lineage that has the same chitinase sequence. Unfortunately, the microsatellite analysis (Grandjean et al. 2014) for this sample was unsuccessful, possibly due to a rather low agent level (A3). However, the sequencing result revealed that at least two lineages of *A. astaci* infected the investigated specimens independently. For the remaining infected crayfish in this study, the *A. astaci* lineage remained unclear. This was due to the low amount of *A. astaci* DNA in most of the samples, which compromises identification of the

*A. astaci* lineage. For the infected specimens in our study, it cannot be concluded at which stage they got infected, whether before or after entering Germany. In any case, horizontal transmission of the disease may represent a serious problem in the crayfish aquarium trade (Mrugała et al. 2015) because it can facilitate the spread of *A. astaci*.

Of the species investigated in our study, only the signal crayfish, one of the Old NICS, has so far established populations in the wild in Europe and is also the most widespread NICS in Europe (Holdich et al. 2009). However, the availability of ornamental crayfish in Europe increases the probability of wild population establishment for other alien crayfish species (Chucholl 2013) and thus the establishment of novel *A. astaci* reservoirs in the wild. Even if the release of individual crayfish does not lead to population establishment, one individual infected with *A. astaci* (like the *P. llamasii* in this study with agent level A6) is a threat to indigenous crayfish species. Thus, uncontrolled spread of *A. astaci* throughout Europe is facilitated by the lack of import restrictions for exotic species, both into and between EU countries. Ireland and Scotland are two cases with strict national alien species policy, as the crayfish aquarium trade is completely banned and the keeping of alien crayfish is illegal (Peay 2009). However, these import restrictions are only effective if the existing laws are also enforced, which seems to be a problem, for example in Ireland, where the parthenogenetic marbled crayfish is available for sale in the pet trade (Faulkes 2015a). An alternative approach to the complete ban of live crayfish imports was proposed by Padilla and Williams (2004). They recommend the posting of bonds to ensure that the costs to repair damage and implement conservation measures that arise from the aquarium trade are covered by those who cause the problems, i.e. importers and traders of crayfish. A similar regulation is implemented in EU regulation 1143/2014, called the polluter pays principle, stating that the costs that arise from the introduction of alien species into Europe are the traders' responsibility.

Our study highlights the potential threats of alien invasive species and the diseases they might be carrying (Holdich et al. 2009; Savini et al. 2010), especially in the case of the public having easy access to pet animals and the opportunity to release them into nature (Chucholl 2013; Mrugała et al. 2015). A serious concern is the pet status of alien crayfish. Pets associated with emotional attitudes are not treated like invasive alien species, as dispensable individuals would not be terminated but released into the wild to continue their life. This constitutes a factor often ignored when analyzing risks of pet

crayfish, as e.g. with the FI-ISK score (e.g., Tricarico et al. 2010; Patoka et al. 2014). One should never underestimate the threat of the pet animals to the receiving ecosystem.

To conclude, we want to emphasize the threat that the crayfish aquarium trade may pose for nature conservation, since the health status of crayfish kept in private aquaria or tank systems is currently largely unknown. We identified nine new crayfish species as carriers and thus potential transmitters of *A. astaci*. Crayfish that are bought from hobby breeders could in many cases be *A. astaci* carriers, and thus an ecosystem hazard and threat to native European crayfish. This is especially true for imported species from North America. Import restrictions for live crayfish trade alone are not sufficient to decrease the uncontrolled spread of *A. astaci*: the education of owners and retailers may also be necessary (Faulkes 2015b) to reduce the threat that the crayfish pet trade poses to indigenous crayfish species. As Souty-Grosset et al. (2016) stated, the trade regulation for NICS has to be of EU-level concern rather than single countries. Five NICS have very recently been included in the list of alien invasive species of Union concern (EU regulation 2016/1141) with the aim to restrict the import and further spread of these species in the European Union. Because crayfish trade through the internet is not easily regulated, the EU aims at establishing early detection surveillance systems for invasive species.

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## Supplementary material

The following supplementary material is available for this article:

**Table S1.** The agreement between with the morphologically determined species and the sequenced DNA fragment as available from GenBank and the closest matching taxon as available from GenBank.

This material is available as part of online article from:

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