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First evidence of crayfish plague agent in populations of the marbled crayfish (*Procambarus fallax* forma *virginalis*)

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ABSTRACT

Key-words: *Marbled crayfish, crayfish plague agent, exotic pathogen, invasive species, real-time PCR*

The introduction of non-indigenous species and associated diseases can cause declines in indigenous flora and fauna and threaten local biodiversity. The crayfish plague pathogen (*Aphanomyces astaci*), carried and transmitted by latent infected North American crayfish, can lead to high mortalities in indigenous European crayfish populations. Although the parthenogenetic marbled crayfish (*Procambarus fallax* (Hagen, 1870) forma *virginalis*) is common in the aquarium trade and has established wild populations in Europe, its carrier status is still unknown. This study investigated one captive and three established wild-living marbled crayfish populations for an infection with the crayfish plague pathogen applying real-time PCR. We demonstrate that captive, as well as two wild marbled crayfish populations were infected by *A. astaci*. Although infection status in laboratory kept specimens reached high levels, marbled crayfish showed no obviously plague-related mortality. Furthermore, sequence analysis revealed that captive crayfish carried the *A. astaci* genotype Pc, which has earlier been isolated from the North American red swamp crayfish (*Procambarus clarkii*). The results indicate that due to its positive carrier status marbled crayfish poses a greater threat to local biodiversity in Europe than considered until now.

RÉSUMÉ

Première mise en évidence de l'agent de la peste de l'écrevisse dans des populations de l'écrevisse marbrée (*Procambarus fallax* f. *virginalis*)

Mots-clés : *écrevisse marbrée, agent de la peste de l'écrevisse, agent pathogène exotique, espèce envahissante,*

L'introduction d'espèces non-indigènes et des maladies associées peut entraîner le déclin de la flore et la faune indigènes et menacer la biodiversité locale. L'agent pathogène de la peste des écrevisses (*Aphanomyces astaci*), porté et transmis par des écrevisses nord-américaines à infection latente, peut conduire à de fortes mortalités chez des populations d'écrevisses européennes indigènes. Bien que l'écrevisse marbrée parthénogénétique (*Procambarus fallax* (Hagen, 1870) forma *virginalis*) soit fréquente dans le commerce d'aquariophilie et qu'elle ait établi des populations sauvages en Europe, son statut de porteur est encore inconnu. Cette étude a porté sur une population captive et trois populations d'écrevisses marbrées sauvages établies pour rechercher une infection par l'agent pathogène

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PCR en temps réel

de la peste des écrevisses en appliquant la PCR en temps réel. Nous démontrons que deux populations d'écrevisses marbrées sauvages ainsi que celle en captivité étaient infectées par *A. astaci*. Bien que l'infection en laboratoire présente des spécimens atteints à des niveaux élevés, les écrevisses marbrées ne présentent pas de mortalité évidente liée à la peste. En outre, l'analyse des séquences a révélé que les écrevisses captives portent le génotype Pc d'*A. astaci*, qui a été précédemment isolé de l'écrevisse rouge des marais nord-américaine (*Procambarus clarkii*). Les résultats indiquent qu'en raison de son statut de porteur l'écrevisse marbrée constitue une plus grande menace pour la biodiversité locale en Europe que considéré jusqu'à présent.

INTRODUCTION

From the end of the 19th century and onwards non-indigenous crayfish species (NICS) from North America, like spiny-cheek crayfish (*Orconectes limosus*), signal crayfish (*Pacifastacus leniusculus*) and red swamp crayfish (*Procambarus clarkii*), were introduced in Europe (Alderman, 1996; Holdich *et al.*, 2009). Besides unintended introductions, *e.g.* by escaping pet specimen, the main reason for their importations was intentional stockings (Lilley *et al*., 1997). For example, the signal crayfish was stocked into natural waters to replace indigenous noble crayfish (*Astacus astacus*) populations, which have been lost because of crayfish plague outbreaks (Vennerström *et al.*, 1998). Although the intentional release of NICS today is restricted in most European countries, the three above mentioned "Old NICS" (*i.e.* NICS introduced before 1975) have already established numerous populations throughout Europe (Holdich *et al.*, 2009; Kouba *et al*., 2014). Presently, a wide variety of different North American crayfish are popular as pets in the aquarium trade, especially in Germany and the Netherlands, and the extensive trade of crayfish species as pets leads to further releases of these animals into nature from aquaria (Chucholl, 2013) and garden ponds (Patoka *et al*., 2014). Despite that scientist promote a ban on the supply and keeping of NICS in aquaria as pets, crayfish availability via aquarium trade, fairs, and internet sales is still increasing in many European countries (Chucholl, 2013).

One of the common crayfish species found in the North American and European pet trade is the marbled crayfish (*Procambarus fallax* (Hagen, 1870) forma *virginalis*) (Faulkes, 2010). It was introduced into Germany via pet trade in the mid 1990s (Chucholl and Pfeiffer, 2010) and became popular due to its extraordinary reproduction strategy, the apomictic parthenogenesis (Scholtz *et al*., 2003; Martin *et al*., 2007, 2010). The first wild-living marbled crayfish were found in 2003 in a dredging pool near Eggenstein-Leopoldshafen (near Karlsruhe, Germany) only one km away from the River Rhine, but without any surface water connection to the river (Marten *et al*., 2004). Today at least six wild established populations are known, most of them from Germany (Chucholl *et al*., 2012). In Europe, the species has also been reported in the wild occurring in Sweden (Bohman *et al*., 2013), the Netherlands, Italy and Slovakia (Kouba *et al*., 2014). Once established, NICS are often more competitive than indigenous crayfsh species (Schulz *et al*., 2006) but they also displace indigenous crayfish species due to associated diseases carried by them (Holdich *et al.*, 2009).

North American crayfish are natural hosts and carriers of the crayfish plague pathogen, the oomycete *Aphanomyces astaci* (Unestam and Weiss, 1970; Alderman, 1996). *A. astaci* is a crayfish parasite (Unestam, 1969) but can also infect two crab species (*Eriocheir sinensis* and *Potamon potamios)* (Svoboda *et al*., 2014; Schrimpf *et al*., 2014). The cysts of the pathogen can survive in freshwater for a few days or in mud for a couple of weeks and give rise to new zoospores which infect the animals (Cerenius and Söderhäll, 1985; Longshaw, 2011). Although North American crayfish are usually resistant to this pathogen and only act as carriers, they can succumb and die from the infection under stressful conditions (Söderhäll and Cerenius, 1992). In contrast, indigenous European crayfish are highly vulnerable and an infection usually leads to high mortality rates (Longshaw, 2011). Today five genotypes of *A. astaci*,

which can be assigned to different host species, are known and they seem to vary in their virulence (Makkonen *et al*., 2012b; Viljamaa-Dirks *et al*., 2013). Genotypes Ps1 and Ps2 have been assigned to signal crayfish (Huang *et al*., 1994), Pc to red swamp crayfish (Diéguez-Uribeondo *et al*., 1995) and Or has been identified on spiny-cheek crayfish (Kozubíková *et al*., 2011). Genotype As has only been identified on native European crayfish species, but until now the original host species in Europe is unknown (Makkonen *et al*., 2012a; Viljamaa-Dirks *et al*., 2013). The genotypes of infected calico crayfish (*Orconectes immunis*) as well as virile crayfish (*Orconectes virilis*), that have been found to be carrier of *A. astaci* (Schrimpf *et al*., 2013a; Tilmans *et al*., 2014), are not yet identified.

Although Culas (2003) had claimed to have detected *A. astaci* DNA in two marbled crayfish specimen (Culas, 2003), her results cannot be regarded as reliable because a later work has shown that the applied PCR method is not specific for *A. astaci*, instead also related *Aphanomyces*-species show a positive signal (Oidtmann *et al.*, 2006). Hence, the carrier status of the parthenogenetic marbled crayfish is still unknown. Therefore, the intention of this study was to investigate captive and wild-living marbled crayfish populations for an infection with the crayfish plague pathogen applying species-specific and quantitative real-time PCR (qPCR). Furthermore, we aimed to determine the *A. astaci* genotype of infected marbled crayfish using sequence analysis. The species could either carry a yet unknown genotype or be carrier of a known genotype due to overlapping habitats, species contact, as well as impurities and exchange through aquarium trade.

MATERIAL AND METHODS

We captured eleven wild-living marbled crayfish with traps and by hand from the Pond in Klepzig (0.025 ha, Sachsen-Anhalt, Germany), and 28 from the Lake Singliser near Borken (74 ha, Hessen, Germany). From the Lake Moosweiher located near Freiburg in the Upper River Rhine catchment (7.6 ha, Baden-Wuerttemberg, Germany), where marbled crayfish is coexisting with spiny-cheek crayfish, we collected 23 marbled crayfish and 28 spiny-cheek crayfish. In Lake Singliser, the marbled crayfish presence was first suspected in October 2010 and one year later, in October 2011, actual proof of its occurrence had been delivered. Being of unknown origin, it was presumed that the animals were released from an aquarium (Dümpelmann and Bonacker, 2012). The first confirmation of a marbled crayfish presence in Lake Moosweiher was provided in July 2009 (Pfeiffer, 2010). Although the lake already contained an established population of spiny-cheek crayfish, marbled crayfish also established a population in Lake Moosweiher. Also here the origin of the population is unknown. Furthermore, 33 marbled crayfish were obtained from a lab culture at Alterra, Wageningen (The Netherlands). The culture was already in-house for approximately six years after founding individuals were purchased from a hobby breeder.

DNA was extracted from the soft abdominal cuticle, the inner joint of two walking legs and parts of the uropods using a CTAB-method as described in Vrålstad *et al*. (2009). To assess the infection status of marbled crayfish, we conducted a TaqMan® minor groove binder (MGB) qPCR, targeting the ITS region according to Vrålstad *et al.* (2009) with some modifications (Schrimpf *et al*., 2013a). Infection status and agent level from the *A. astaci*-specific qPCR are based on the numbers of observed PCR forming units (PFU) and were defined according to Vrålstad *et al*. (2009). DNA samples with an agent levels of A2 ($5\ \text{PFU} \leqslant \text{PFU}_{obs} < 50\ \text{PFU}$) and higher (A3: $50\ \text{PFU} \leqslant \text{PFU}_{obs} < 10^3\ \text{PFU}$; A4: $10^3\ \text{PFU} \leqslant \text{PFU}_{obs} < 10^4\ \text{PFU}$; A5: $10^4\ \text{PFU} \leqslant \text{PFU}_{obs} < 10^5\ \text{PFU}$) are considered infected with *A. astaci* and samples with A0 (0 PFU) and A1 ($\text{PFU}_{obs} < 5\ \text{PFU}$) are considered uninfected. *A. astaci* prevalence in marbled crayfish populations as well as 95% confidence intervals were estimated according to Filipová *et al*. (2013) using the function "epi.conf" (included in package epiR) with RStudio version 0.98.501.

The genotype of *A. astaci* was identified using sequence analysis of a 370 base pair fragment of the chitinase gene according to Makkonen *et al*. (2010) with some modifications. We have used 5× PCR buffer, 2 μM $MgCl_2$, 0.025 u TaqMan® Taq (all Promega, Mannheim, Germany), 0.2 μM dNTP (Fermentas, St. Leon-Rot, Germany), 0.2 μM primers AAChiF and

Table I
qPCR results for one laboratory cultured marbled crayfish population, two wild-living marbled crayfish population and one coexisting population of marbled crayfish (MC) and spiny-cheek crayfish (SC) from sampling sites in Germany. Shown is the number (N) of tested individuals, their detected agent level (A0 to A5, considering A0 (0 PFU) and A1 ($PFU_{obs} < 5$ PFU) uninfected and A2 (5 PFU $\leqslant PFU_{obs} < 50$ PFU), A3 (50 PFU $\leqslant PFU_{obs} < 10^3$ PFU), A4 (10^3 PFU $\leqslant PFU_{obs} < 10^4$ PFU) and A5 (10^4 PFU $\leqslant PFU_{obs} < 10^5$ PFU) infected), the absolute number of infected individuals, A. astaci prevalence in different marbled crayfish populations as well as 95% confidence intervals.

Origin	Species	*N*	Agent level						Infected		95% CI
			A0	A1	A2	A3	A4	A5	*N*	%	
Laboratory cultured	MC	33	9	1	16	5	1*	1*	23	70	**(53–83%)**
Klepzig	MC	11	8	2	1				1	9	**(2–38%)**
Lake Moosweiher	MC	23	16	5	2				2	9	**(2–27%)**
Lake Moosweiher	SC	28	20	7	1				1	4	**(1–18%)**
Lake Singliser	MC	28	28						0	0	**(0–12%)**

*Samples used for the sequence analysis.

AAChiR and added 1.5 μL template DNA for a final volume of 12.5 μL. PCR products were sequenced on a 3730 DNA Analyzer eight capillary sequencer (Applied Biosystems, MA, USA). The sequences were edited with the program Geneious R7 (Drummond *et al*., 2011) and submitted to GenBank (accession number: KP100541). Reference sequences of genotypes As, Ps1, Ps2, Pc were received from J. Makkonen (University of Eastern Finland) and a reference sequence of the genotype group Or was generated from a pure culture sample (strain Li05, isolated from *O. limosus* from the stream Litavka, see Kozubíková-Balcarová *et al*. (2013)) received from A. Petrusek (Charles University in Prague), respectively. Multiple alignments with our sequences were created and the genotype was determined by comparison of our sequences to the reference sequences. Based on the matching mutations the genotypes could be assigned.

RESULTS

The results from the qPCR revealed that 23 out of 33 (70%) marbled crayfish individuals from the lab culture were infected with *A. astaci* (Table I). Agent levels ranged from A2 to A5. In the marbled crayfish population from Lake Singliser, however, no traces of *A. astaci* DNA could be detected, while in the population from Klepzig one of eleven marbled crayfish (9%) was infected with *A. astaci*. In the population from Lake Moosweiher two of 23 marbled crayfish (9%) were *A. astaci* positive as well as one of 28 (4%) of the coexisting spiny-cheek crayfish. All positive crayfish individuals collected from the wild yielded only agent level A2, corresponding to very low agent levels. Since the detection of the *A. astaci* genotype is rarely possible for agent levels lower or equal to A3 (Makkonen *et al.*, 2012a) only for two of the samples from the lab culture with agent level A4 and A5 the genotype of *A. astaci* could be determined. This revealed that the laboratory cultured individuals were carrying the Pc-genotype.

DISCUSSION

This study revealed that marbled crayfish in captivity as well as in nature were infected with the crayfish plague agent. While wild-living individuals showed a low agent level (A2), caged marbled crayfish were more heavily infected, up to agent level A5 (Table I). The high agent levels of the marbled crayfish from the lab culture might be explained by the additional stress caused by the indoor situation and the captivity (dense population, restricted space) which weakens their immune system (Söderhäll and Cerenius, 1992) and facilitates the spread of *A. astaci* in the crayfish body. Moreover, the successful spread of *A. astaci* zoospores rises

with increasing density of crayfish (Kozubíková *et al*., 2009) and the capability of the crayfish to cope with the infection decreases with increasing spore density (Oidtmann, 2012). Strand *et al*. (2011) have detected higher density of spores in indoor tanks, compared to outdoor ponds where spores are heterogeneous distributed due to higher spore dynamics, patchy distributed crayfish and more water per crayfish and *A. astaci* spores. Although no obvious signs of a crayfish plague infection (*e.g.* high mortality, lack of coordination, loss of escape reflex (Alderman *et al.*, 1987; Oidtmann, 2012)) were observed, the zoospore density in the aquarium might be higher than in nature and could lead to higher agent levels of marbled crayfish.

In two out of three wild-living marbled crayfish populations a low *A. astaci* infection could be verified. However, the number of samples was relatively small (N = 11 to 28) and due to environmental stochasticity (Dwyer *et al*., 2004) or recent molting events (Oidtmann, 2012), the infection status with *A. astaci* may have been underestimated. The analysis of 28 individuals seems to indicate that the population from Lake Singliser is *A. astaci* free (Schrimpf *et al*., 2013b). However, a more intensive sampling could possibly still increase detection probability (95% confidence interval ranged from 0 to 12%) of *A. astaci*.

The OIE (2012) recommends either a conventional PCR analysis followed by the sequence analysis of the ITS-region or the qPCR by Vrålstad *et al*. (2009) to confirm the crayfish plague agent in tissue material. We have applied the more sensitive qPCR. As an additional confirmation we have sequenced a fragment of the chitinase gene according to Makkonen *et al*. (2010) and compared it to reference sequences of *A. astaci* from pure culture. The comparision served as species identification as well as genotype assignment.

Interestingly the captive marbled crayfish from the aquarium carried the known genotype Pc, which has earlier been isolated from the warm-water favouring North American red swamp crayfish. In addition, the assigned genotype Pc is physiologically adapted to warm temperatures (Diéguez-Uribeondo *et al.*, 1995) and the spores, compared to other strains (4–20 °C), grow better at higher temperatures up to 27 °C (Oidtmann, 2012). Altough marbled crayfish also prefer warm habitats with water temperatures >15 °C (Chucholl and Pfeiffer, 2010), we cannot differentiate if marbled crayfish originally carried genotype Pc or if the population became infected with this genotype due to contaminations in the aquarium trade or the laboratory. The latter is a possibility, since other crayfish species were maintained in the same laboratory room as well. Although there was no water flow from one tank to another, cross-contaminations while feeding or handling the animals cannot be excluded. Unfortunately, the detection of the *A. astaci* genotype was not possible in samples with lower agent levels from the wild populations. The failure to determine the genotype of low infected crayfish by chitinase sequencing analysis has been frequently observed (Makkonen, person. comm.) and could be explained by the lower sensibility of the traditional PCR compared to qPCR (Tuffs and Oidtmann, 2011) and the lower copy number of the chitinase gene compared to the multycopy-gene ITS targeted in the qPCR. In Lake Moosweiher marbled crayfish coexist with spiny-cheek crayfish for at least five years (Chucholl and Pfeiffer, 2010) and on spiny-cheek crayfish from the Czech Republic the genotype Or has been identified in the past (Kozubíková *et al*., 2011). Thus, if the source of the *A. astaci* infection in marbled crayfish was spiny-cheek crayfish, we would expect both species to carry genotype Or. But it is also possible that the spiny-cheek crayfish population was uninfected and got infected from marbled crayfish. Further investigations might clarify the situation.

Infected non-indigenous crayfish usually act as permanent reservoirs of *A. astaci* and once such a reservoir is present in nature, the pathogen can infect other indigenous and non-indigenous populations through contaminated water, fishing gear or animals (Oidtmann, 2012). Besides transmitting crayfish plague, marbled crayfish can also threat indigenous European species by competition for resources and high reproduction rates due to high growth rate and early fertility (Marten *et al*., 2004). Marbled crayfish favor summer-warm lentic habitats with water temperatures >15 °C. Hence, it is expected that marbled crayfish can reproduce and establish stable populations, in addition to recent distribution areas (Germany, Sweden, the Netherlands, Italy and Slovakia), in France, parts of England, Eastern Europe,

as well as the Iberian and the Balkan Peninsula, including the potential to coexist with other North American species (Chucholl and Pfeiffer, 2010). Although marbled crayfish could establish viable populations in these areas, environmental factors (*e.g.* food sources) often limit the population growth and spread in nature (Marten *et al*., 2004).

According to Holdich *et al.* (2009) "New NICS" can be more easily controlled, managed or eliminated than the more common "Old NICS" because the chance to control a species is higher when the species is not yet widespread. Altough the eradication of a restricted, isolated signal crayfish population in a small pond using chemical treatment, pharmaceutical BETAMAX VET®, and draining appeared to be successful, the application to larger water systems will probaly not have the same success (Sandodden and Johnsen, 2010). Furthermore, with a chemical treatment it cannot be assured that only the target species will be eliminated. Therefore, we cannot consider the chemical treatment as a save way to completely control invasive crayfish species. Especially the control and eradication of parthenogenetic species that only need one female to establish viable populations (Marten *et al*., 2004) might be a challenge in future.

Since their introductions "Old NICS" from North America, *e.g.* signal crayfish, spiny-cheek crayfish and red swamp crayfish, became the greatest threat to indigenous crayfish species due to transmission of crayfish plague and habitat loss as a result of direct competition (Holdich *et al*., 2009). Hence, the marbled crayfish is a much greater menace than previously known, because our results confirm that this highly reproductive species is infected with the crayfish plague agent. Therefore, there is an urgent need to minimize the risk of further introductions of marbled crayfish into nature. Conservation measures including the education of pet traders, local stakeholders and fisher man is one important measure in halting the further spread of NICS.

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