Apparent interspecific transmission of *Aphanomyces astaci* from invasive signal to virile crayfish in a sympatric wild population

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Abstract

The crayfish plague pathogen (*Aphanomyces astaci*) causes mass mortalities of European crayfish when transmitted from its original North American crayfish hosts. Little is known, however, about interspecific transmission of the pathogen between different American crayfish species, although evidence from trade of ornamental crayfish suggests this may happen in captivity. We screened signal and virile crayfish for *A. astaci* at allopatric and sympatric sites in a UK river. Whilst the pathogen was detected in signal crayfish from both sites, infected virile crayfish were only found in sympatry. Genotyping of *A. astaci* from virile crayfish suggested the presence of a strain related to one infecting British signal crayfish. We conclude that virile crayfish likely contracted *A. astaci* interspecifically from infected signal crayfish. Interspecific transmission of *A. astaci* strains differing in virulence

between American carrier species may influence the spread of this pathogen in open waters with potential exacerbated effects on native European crayfish.

Keywords: *Pacifastacus leniusculus*; *Orconectes* cf. *virilis*; Wildlife disease; Transmission pathways.

1. Introduction

The crayfish plague agent, *Aphanomyces astaci*, is arguably one of the most devastating invasive parasites in European freshwaters (Lowe, 2004; DAISIE, 2009). Since its first introduction in the mid-19th century (Alderman, 1996; Holdich, 2003), the pathogen has spread throughout Europe, facilitated in recent decades by movements of invasive North American (henceforth referred to as American) crayfish (Souty-Grosset *et al.,* 2006; Holdich *et al.,* 2014; James *et al.,* 2014). Whilst American crayfish are often asymptomatic carriers of *A. astaci* infection, the disease is usually lethal in European species (Unestam & Weiss, 1970; Diéguez-Uribeondo *et al.,* 1997; Bohman, *et al.,* 2006; Kozubíková *et al.,* 2008). Once introduced, *A. astaci* can spread rapidly, transmitted through zoospores that are released into water (Oidtmann *et al.,* 2002) and can survive for at least 14 days (CEFAS, 2000). Spores are mainly released during host moulting or death (Svoboda *et al.,* 2013), and within a cadaver *A. astaci* can remain viable for several days (Oidtmann *et al.,* 2002). Therefore, the movement of infected carcasses by predators could facilitate pathogen dispersal. If fish ingest infected tissue, the pathogen can even survive passage through the gastro-intestinal tract, providing an additional transmission pathway (Oidtmann *et al.,* 2002).

Whilst the transmission of *A. astaci* from non-native American to European crayfish has been widely documented (e.g. Alderman *et al.,* 1990; Diéguez-Uribeondo, Temiño & Múzquiz, 1997; Vennerström *et al.,* 1998; Bohman *et al.,* 2006), little is known about

interspecific pathogen transmission between these invasive carriers. Until now, four different *A. astaci* genotype groups have been isolated in Europe; group A was obtained from infected native European crayfish (*Astacus astacus* and *A. leptodactylus*) and groups B, D and E from different American crayfish species (*Pacifastacus leniusculus*, *Procambarus clarkii* and *Orconectes limosus*, respectively) (Svoboda *et al.*, 2017). The genotype groups infecting additional *A. astaci* carriers known from European waters, calico (*Orconectes immunis*), marbled (*Procambarus fallax* f. *virginalis*) and virile (*Orconectes* cf. *virilis*) crayfish (Filipova *et al.*, 2013; Schrimpf *et al.*, 2013; Keller *et al.*, 2014; Tilmans *et al.*, 2014), are so far unknown. Existing data suggest that *A. astaci* genotype groups are host-specific among American crayfish (Grandjean *et al.*, 2014). There is no evidence of strains transmitting between these crayfish in the wild, although it seems to occur in the aquarium trade (Mrugała *et al.*, 2015).

Here, we investigate interspecific transmission of *A. astaci* upon contact of two potential carrier species. Signal crayfish are widespread across the UK (James *et al.*, 2014) and were initially stocked into the River Lea during the mid-1970s (Almeida *et al.*, 2014). Conversely, virile crayfish are only found in the Lea catchment in the UK (James *et al.*, 2014), possibly unintentionally introduced there around 2004 (Ahern *et al.*, 2008). The two species have been co-existing since at least 2011 (James *et al.*, 2015). Virile crayfish in this river, as well as in a population from the Netherlands, have been reported to carry *A. astaci* (Tilmans *et al.*, 2014), and it was suggested that these crayfish were already infected prior to introduction. Here, we tested this hypothesis, the alternative hypothesis that virile crayfish contracted *A. astaci* from co-existing signal crayfish, by evaluating the distribution of the pathogen at allopatric and sympatric sites within the River Lea and an adjacent lake in London, and by genotyping the pathogen from infected host specimens.

2. Methods

Invasive signal crayfish and virile crayfish were collected from the River Lea and an adjacent lake in London, UK, during September 2014. Using baited traps employed over two consecutive nights and checked daily, animals were caught from allopatric (Lat/Long: 51°45′14″N/ 000°00′16″E, 51°42′29″N/ 000°01′16″W for signal and virile crayfish respectively, n = 30 for each species) and a sympatric site (Lat/Long: 51°42′24″N/ 000°01′04″W, n = 9 signal and 30 virile crayfish) (Fig. 1). Upon capture, animals were transported individually to Cardiff University (UK), humanely euthanized by freezing at -80^{o}C and stored in ca. 95% molecular grade ethanol before transport to Charles University in Prague for further processing. For *A. astaci* screening, we harvested from each animal a section of tail fan, soft abdominal cuticle, two limb joints, and any melanised cuticle (as in Svoboda *et al.*, 2014). Tissue samples from each individual (40-50 mg) were ground together in liquid nitrogen from which DNA was extracted using a DNeasy tissue kit (Qiagen) as per manufacturer's guidelines.

All samples were screened for *A. astaci* presence using TaqMan MGB quantitative PCR (qPCR) on the iQ5 BioRad thermal cycler according to Vrålstad *et al.*, (2009), slightly modified to increase assay specificity (Strand *et al.*, 2011; Svoboda *et al.*, 2014). To check for potential inhibition (as in Kozubíková *et al.*, 2011; Svoboda *et al.*, 2014) each DNA isolate was analysed by qPCR at two concentrations (undiluted and 1:10 dilution). Negative controls were included at each step of the protocol, and in all cases these remained negative. Based on the estimated amount of *A. astaci* DNA in the isolates (expressed in PCR-forming units, PFU), the extent of the infection was expressed in semi-quantitative agent levels (A0-A7; according to Vrålstad *et al.*, 2009; Kozubíková *et al.*, 2011). Samples with agent levels of A2 or higher were considered positive for *A. astaci*.

For *A. astaci* genotype group identification, we analysed *A. astaci*-positive samples using nine *A. astaci*-specific microsatellite markers (Grandjean *et al.*, 2014). As amplification success depends on the amount of pathogen DNA in the sample, genotyping was only attempted for those with agent level A3 and higher (as in Grandjean *et al.*, 2014) and was repeated three times for each sample. In case of an initial lack of amplification, DNA isolates were concentrated on the Concentrator Plus 5305 (Eppendorf). The results of successful genotyping were compared with the *A. astaci* reference strains described by Grandjean *et al.*, 2014 and an *A. astaci*-positive DNA isolate from signal crayfish in Lake Mochdre (Newtown) Wales, UK (James *et al.*, 2017).

3. Results

Within allopatric sites on the River Lea, *Aphanomyces astaci* was detected in 83% (25 out of 30) signal crayfish but was not detected in any virile crayfish (n = 30). From the sympatric site, 44% (4 out of 9) signal crayfish and 23% (7 out of 30) virile crayfish tested positive for *A. astaci* infection. All *A. astaci*-positive samples yielded low levels of infection (A2-A3; Vrålstad *et al.*, 2009). Of the *A. astaci* infected animals from the allopatric signal crayfish site A3 level infections were detected in four animals (estimated PFUs: 51, 71, 106 and 111). Within the sympatric site, A3 level infections were detected in three virile (estimated PFUs: 85, 167 and 1000) and two signal (estimated PFUs: 52 and 57) crayfish.

Due to low amount of *A. astaci* DNA, reliable amplification and scoring of the microsatellites were only possible for two specimen of virile crayfish. Of the nine microsatellite loci, amplification was achieved for seven loci for the first virile crayfish specimen, and for six loci for the second one. For the first virile crayfish specimen the multilocus genotype corresponded at five loci to the reference axenic culture of the genotype group B (Table 1). Differences were observed at the Aast9 and Aast12 loci, where

125 homozygotes rather than heterozygotes were scored. Such variation at the Aast9 locus has
126 been also observed in the *A. astaci*-positive DNA isolate from signal crayfish in Wales, UK
127 (Table 1; James *et al.* 2017). For the second virile crayfish specimen the multilocus genotype
128 corresponded at five loci to the reference axenic culture of genotype group B (Table 1). A
129 difference was observed at the Aast9 locus, where a homozygote rather than a heterozygote
130 was scored. All six of the amplified loci for this second specimen matched the *A. astaci*
131 positive DNA isolate from signal crayfish in Wales (Table 1).
132
133 **4. Discussion**
134 Here we present two lines of evidence suggesting the interspecific transmission of
135 *Aphanomyces astaci* between two invasive American crayfish species from the UK. Firstly,
136 virile crayfish were infected at a site in the River Lea where they coexisted with signal
137 crayfish, but were not found to be infected at an allopatric site. In contrast, signal crayfish
138 were infected with *A. astaci* both in regions of sympatry and allopatry. Secondly, the *A.*
139 *astaci* multilocus genotype identified in virile crayfish was similar, although not identical, to
140 the reference strain of the genotype group B, isolated in Europe from infected signal crayfish
141 (Huang *et al.,* 1994; Grandjean *et al.,* 2014), and closely matched the multilocus genotype
142 detected in a UK signal crayfish population (Table 1). Therefore, the current study may
143 represent the first report of *A. astaci* being interspecifically transmitted within a wild, mixed
144 species population of American crayfish in their invasive range.
145 Whilst the multilocus gentotypes obtained for the *A. astaci*-positive DNA isolates
146 from virile crayfish in the River Lea were not an identical match to the reference strain of the
147 group B from Europe, such within group variation has already been reported (Grandjean *et*
148 *al.,* 2014; Maguire *et al.* 2016; Mrugała *et al.,* 2016). This, combined with the fact that the *A.*
149 *astaci* genotype obtained from infected virile crayfish was not similar to any of the other four

genotype groups currently described (Grandjean *et al.,* 2014), make it a likely assumption that the virile crayfish in this study harboured an *A. astaci* strain from the genotype group B pathogen. Of all American crayfish species, this genotype group has only previously been recorded from wild signal crayfish. In addition, the multilocus genotypes obtained from both virile crayfish specimens closely matched that isolated from signal crayfish elsewhere in the UK. Variation detected at the locus Aast 12 may have resulted inefficient amplification of low concentration DNA template). Both virile crayfish specimens also shared a homozygote pattern at locus Aast 9 in contrast to the heterozygote reported in the original reference sequence (James *et al.*, 2017). Combined these data add to the evidence that virile crayfish likely contracted *A. astaci* from infected signal crayfish within the UK.

Possessing a wide host range is one of the key factors in determining the success of an introduced parasite (Kennedy 1994). It has been speculated that the interspecific transmission of *A. astaci* genotypes between American crayfish species may be limited by host-pathogen incompatibilities (Tilmans *et al.,* 2014). Our findings cast doubt on this hypothesis, and highlight the need for greater investigation into the host range of different *A. astaci* genotype groups. Given that *A. astaci* genotypes different in their virulence (Makkonen *et al.,* 2012, 2014; Viljamaa-Dirks *et al.,* 2013) and climate requirements (Diéguez-Uribeondo *et al.,* 1995; Rezinciuc *et al.,* 2014) interspecific transmission may influence the spread of the pathogen across Europe. This is likely to have implications for native crayfish conservation given that *A. astaci* affords American crayfish species an even greater competitive advantage over their susceptible European counterparts. Interspecific transmission may also result in complex mixed genotype group infections with unknown consequences for both American carrier crayfish and native European crayfish hosts. Ultimately, this may also influence pathogen evolution.

This study provides the first indication of *A. astaci* being transmitted between American carrier crayfish species in a wild population. We do, however, acknowledge the limitations of our study in terms of incomplete genotyping and low sample size. Studies of *A. astaci* interspecific transmission are particularly relevant given that many European countries now harbour multiple non-native American crayfish species (Kouba *et al.,* 2014). With microsatellite markers allowing the characterization of *A. astaci* genotypes from host tissue samples, increased effort should be focused into genotyping the pathogen strains infecting American carrier crayfish species. Characterising the strain(s) of *A. astaci* responsible for a crayfish plague outbreak may also help identify the source of an epidemic and monitor the spread of infection (see Kozubíková *et al.,* 2014, Vrålstad *et al.,* 2014).

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Figure 1. Sample sites along the River Lea (UK) and an adjacent lake for invasive signal (*Pacifastacus leniusculus*) and virile (*Orconectes* cf. *virilis*) crayfish. River flow direction is indicated by the black arrow. Allopatric sites shown for signal (●) and virile crayfish (▲) respectively, and the sympatric site (■). Note: the location of the allopatric virile crayfish site is a lake adjacent to the river. More details about virile and signal crayfish distribution in this region can be found in James *et al.*, (2016). Image courtesy of Maps data 2016 @Google.

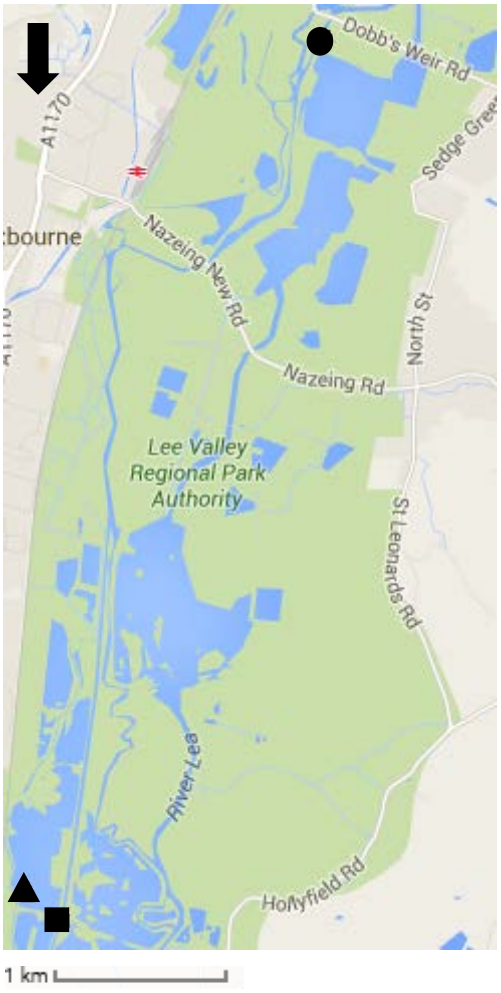

Table 1. Comparison of allele sizes of nine microsatellite loci from the original European reference strain of *Aphanomyces astaci* genotype group B (Grandjean *et al.*, 2014), a reference strain from *Pacifastacus leniusculus* from Wales, UK (James *et al.*, 2017) and *A. astaci*-positive samples of *Orconectes* cf. *virilis* examined in the current study.

Locus	**Original European reference strain VI03555 (*P. leniusculus*)**	**UK population (*P. leniusculus*)**	**UK population (*O.* cf. *virilis*) specimen 1**	**UK population (*O.* cf. *virilis*) specimen 2**
Aast 2	142	142	142	142
Aast 4	87	87	87	n/a
Aast 6	148	148	n/a	148
Aast 7	215	215	215	n/a
Aast 9	164/182	164	164	164
Aast 10	132	132	n/a	132
Aast 12	226/240	226/240	240	226/240*
Aast 13	202	202	202	202
Aast 14	248	248	248	n/a

*both alleles were scored but respective peaks were observed in separate PCR runs. n/a – loci with no amplification, likely due to low concentration of *A. astaci* DNA in the isolate.