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The prevalence of *Aphanomyces astaci* in invasive signal crayfish from the UK, and implications for native crayfish conservation

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Running title: *Aphanomyces astaci* in the UK

Summary

The crayfish plague agent, *Aphanomyces astaci*, has spread throughout Europe, causing a significant decline in native European crayfish. The introduction and dissemination of this pathogen is attributed to the spread of invasive North American crayfish, which can act as carriers for *A. astaci*. As native European crayfish often succumb to infection with *A. astaci,* determining the prevalence of this pathogen in non-native crayfish is vital to prioritise native crayfish populations for managed translocation. In the current study, 23 populations of invasive signal crayfish (*Pacifastacus leniusculus*) from the UK were tested for *A. astaci* using quantitative PCR. Altogether, 13 out of 23 (56.5%) populations were found to be infected, and pathogen prevalence within infected sites varied from 3 to 80%. Microsatellite pathogen genotyping revealed that at least one UK signal crayfish population was infected with the *A. astaci* genotype group B, known to include virulent strains. Based on recent crayfish distribution records and the average rate of signal crayfish population dispersal, we identified one native white clawed crayfish (*Austropotamobius pallipes*) population predicted to come into contact with infected signal crayfish within five years. This population should be considered as a priority for translocation.

Key words: Pacifastacus leniusculus; *crayfish plague*; *white clawed crayfish*; Austropotamobius pallipes; *Ark Sites*

Introduction

Crayfish plague, caused by the oomycete *Aphanomyces astaci,* is arguably one of the most deadly invasive parasites of freshwater ecosystems worldwide (Lowe et al. 2004; DAISIE 2009). The pathogen is thought to have been first introduced into Europe (Italy) in 1859, and has subsequently spread throughout most of the continent (reviewed by Alderman 1996; Holdich 2003). In the latter half of the 20th century the spread of *A. astaci* throughout Europe was facilitated by the movement of non-native North American (henceforth referred to as American) crayfish (reviewed by Alderman 1996; Holdich 2003). Whilst American crayfish are often asymptomatic carriers of the pathogen, in native European crayfish infection is usually fatal (Unestam and Weiss, 1970; Diéguez-Uribeondo et al. 1997; Bohman et al. 2006; Kozubíková et al. 2008; Oidtmann, 2012). Therefore, preventing the spread of this pathogen in regions with populations of highly susceptible hosts is a conservation priority.

One of the main American crayfish species responsible for spreading *A. astaci* in Europe, the signal crayfish (*Pacifastacus leniusculus*), was first introduced into the UK from Sweden during the 1970s for aquaculture (e.g. Holdich and Reeve 1991; Alderman 1996; Peay and Hiley, 2005; Holdich et al. 1999, 2014). This corresponded with mass declines in Britain's historically abundant native white clawed crayfish (*Austropotamobius pallipes*) (see Holdich and Reeve 1991; Holdich and Sibley 2009; Holdich et al. 2009, 2014; James et al. 2014), to such an extent that since 2010 they have been categorised as endangered (IUCN, 2015). Whilst it was widely considered that reductions in native crayfish were, at least partially, due to the transmission of *A. astaci* from signal crayfish, screening and detection of this pathogen in the UK did not occur until the early 1980s (Alderman 1996). One of the first suspected outbreaks of plague in the UK was recorded from the River Lee, Thames catchment, England in 1981 (Alderman 1996). The pathogen has since been reported in native crayfish from several other sites in England as well as Wales and Ireland (Alderman et

al. 1984, 1990; Holdich and Reeve 1991; Alderman 1996; Lilley et al. 1997; Holdich 2003). However, these reports have been based on pathogen morphology and disease symptoms in native European crayfish. Given that there are no morphological features that distinguish *A. astaci* from non-pathogenic *Aphanomyces* species (Royo et al. 2004; Oidtmann 2012), molecular confirmation is essential (Oidtmann et al. 2006; Vrålstad et al. 2009). The only report in the scientific literature of *A. astaci* detection in the UK using molecular methods is from another introduced crayfish species, *Orconectes* cf. *virilis* (see Tilmans et al. 2014), which is restricted to a single catchment (James et al. 2016).

Gaining a comprehensive understanding of *A. astaci* distribution in the UK is essential for native crayfish conservation. It is generally considered that the only way of ensuring the sustainability of white clawed crayfish in the UK is through the establishment of isolated "Ark Sites" free from non-native crayfish and at low risk of their invasion (Peay, 2009). Resources for implementing such conservation measures are, however, limited and so the selection of native crayfish populations for translocation needs to be a well-informed process. Native crayfish populations in close vicinity to *A. astaci*-infected invasive crayfish populations are at higher risk of extirpation than those neighbouring uninfected ones (Söderbäck, 1994; Westman et al. 2002; Schulz et al. 2006; Dunn et al. 2009; Schrimpf et al. 2013). Co-existence of native crayfish with invasive crayfish for several years has been observed in the absence of *A. astaci* (see Söderbäck, 1994; Westman et al. 2002; Schulz et al. 2006; Dunn et al. 2009; Schrimpf et al. 2013). Therefore, it is of greater urgency to translocate native crayfish populations at high risk of *A. astaci* transmission, than those in close proximity to uninfected invasive crayfish.

Here, we used quantitative PCR (qPCR) to assess the prevalence and intensity of infection with *A. astaci* in 23 populations of invasive signal crayfish in England and Wales. Using these data in combination with long term white clawed crayfish distribution records

(James et al. 2014) we identified native crayfish populations at high risk of infection with *A. astaci* (determined by their proximity to an *A. astaci*-infected signal crayfish population). Given that *A. astaci* genotypes differ in virulence (Becking et al. 2015; Makkonen et al. 2012), when possible, we also genotyped the strain of *A. astaci*.

Methods

For this study, invasive signal crayfish (*Pacifastacus leniusculus*) from the UK were screened for the presence of *Aphanomyces astaci* using similar molecular methods in two separate laboratories; the Centre for Environment, Fisheries and Aquaculture Science, Weymouth, UK (Cefas), and Charles University, Prague, Czech Republic (CUNI). At all sites, signal crayfish were captured from rivers and ponds using baited traps. Upon collection, animals were transported to the laboratory and humanely euthanized by exposure to chloroform vapour or freezing at -80^{o}C, before being stored individually in falcon tubes containing 95% molecular grade ethanol. Samples collected between September 2009-July 2010 from 17 sites were processed at Cefas (n = 8 to 30 animals per site), whereas those harvested during May-September 2014 were analysed at CUNI (n = 20 to 30 animals per site, Table 1).

From each crayfish, a section of tail fan and soft abdominal cuticle were harvested for *A. astaci* screening. For animals processed in CUNI soft cuticle from two limb joints and any sections of melanised cuticle were also collected and pooled (Svoboda et al. 2014). At Cefas tissue samples from the tail fan and soft abdominal cuticle were analysed separately (mean: 60 and 78 mg of tissue per host sample for the tail fan and soft abdominal cuticle respectively). For these samples, tissue disruption was conducted in fast prep tubes containing lysis matrix A (MP Biomedicals) and DNA subsequently extracted using the Qiamp DNeasy Biorobot investigator kit (Qiagen), according to the manufacturer's guidelines. At CUNI, for each animal, all collected tissue samples were amassed (40-50 mg

per host sample) and ground together in liquid nitrogen. DNA was then extracted using DNeasy tissue kit (Qiagen) in accordance with manufacturer's instructions.

All samples were tested for *A. astaci* presence with the TaqMan MGB quantitative PCR (qPCR) as described in Vrålstad et al. (2009) with the following slight alterations at Cefas and CUNI respectively: an elongation of the decontamination step from 2 to 5 min (Tuffs and Oidtmann, 2011), an increase in the annealing temperature from 58-60^{o}C and decreased synthesis time from 60 to 30s (Svoboda et al. 2014). At Cefas and CUNI qPCRs were run on a Step one Plus real time cycler (Applied Biosystems) and an iQ5 BioRad thermal cycler, respectively. Negative controls were used in every step of the procedure; these remained negative in all cases. The amount of A. astaci DNA in each sample was estimated/quantified based on the calibration curve of a set of standards. At Cefas the quantity of pathogen DNA in these standards ranged from 1ng-10fg, in a 10-fold dilution series. At CUNI four standards were included containing 3 x 4^{10}, 3 x 4^{8}, 3 x 4^{4} and 3 x 4^{2} PCR Forming Units (PFU) of pathogen DNA. At Cefas each sample was run in triplicate and an average taken when calculating pathogen DNA concentrations. At CUNI each isolate was run twice, undiluted and a 10-fold diluted replicate to test for inhibition that may affect the efficacy of pathogen detection (Vrålstad et al. 2009; Strand et al. 2011). Based on the strength of the PCR signal, we assigned the relative level of *A. astaci* infection to semi-quantitative agent levels (A0-A7; according to Vrålstad et al. 2009; Kozubíková et al. 2011). Samples designated as A2 or higher were considered positive for *A. astaci* presence. These data were used to determine the prevalence of *A. astaci* in the studied populations, and its 95% confidence intervals. Confidence intervals were calculated using the function "epi.conf" included in the library "epiR" (Stevenson et al. 2013) for the statistical package R vs 3.2 (R, 2013).

Pathogen genotyping was only conducted for *A. astaci*-infected crayfish that were tested at CUNI (samples processed at Cefas were done so before microsatellite genotyping became available for *A. astaci*). As most of these crayfish harboured relatively low infection intensities (A2-A3), pathogen genotyping was only possible for crayfish from one population, the Mochdre Brook (Wales). From this population, pathogen DNA from one crayfish (harbouring an A3 agent level infection) was analysed using nine *A. astaci*-specific microsatellite markers (Grandjean et al. 2014). Genotyping was attempted for another crayfish from this population but, presumably due to the relatively small amount of pathogen DNA present, this was un-successful. Prior to genotyping the sample was concentrated using a Concentrator Plus 5305 (Eppendorf) to increase pathogen DNA concentration. The results were compared with the *A. astaci* reference strains described in Grandjean et al. (2014).

We assessed native white clawed crayfish populations at potential risk from the 13 signal crayfish populations where we detected *A. astaci* using recent (2009 onwards) native crayfish distribution records (Craybase database, James et al. 2014). In this regard, we are aware that it is not possible to declare those signal crayfish populations where the pathogen was not detected as uninfected. As such it should be noted that in the context of native crayfish risk assessment the purpose of this study is only to show where *A. astaci* definitely is present (or has been recently) and highlight surrounding native crayfish populations potentially at risk of disease. For these purposes, sites where *A. astaci* was detected were mapped and any native crayfish populations, not already exposed to signal crayfish, within a 7.5, 10, 12 or 15 km aerial radius were recorded. Locations harbouring native crayfish were searched for within the signal crayfish records contained in Craybase (7166 in total, James et al. 2014), and only those not already invaded were considered for risk assessment purposes. Buffer zones (i.e. 7.5, 10, 12 and 15 km) were selected on the basis that the average rate of signal crayfish population expansion along a river in the UK has been estimated as 1.5 km

per year (Bubb et al. 2004; although it should be acknowledged that the rate of signal crayfish dispersal is faster in other European countries e.g. Hudina et al. 2009; Weinländer and Füderer, 2009). Therefore, we presume that populations within 7.5 km of each other are predicted to come into contact within 5 years, providing that they inhabit connected waterbodies. These analyses were performed using ArcGIS version 10.3 mapping software.

Results

Aphanomyces astaci was detected in 56.5% (13 out of 23) signal crayfish populations from Wales and England (Table 1, Figure 1). Among infected populations, prevalence ranged from 3-80% at generally low infection intensities (agent levels A2-A3) with the exception of Mochdre Brook in Wales, and Bently Brook and River Lee in England (Table 1, Figure 1). DNA (i.e., agent levels A1) were detected in tested isolates from single crayfish specimens (Table 1, Figure 1). The agent level A1 should not considered a reliable detection of *A. astaci* (Vrålstad et al. 2009) but such observations should raise concern about its potential presence in the tested population. A multilocus microsatellite genotype of *A. astaci* was only obtained from the Mochdre Brook signal crayfish population. This was identical to the reference axenic culture of the genotype group B strain at eight loci, but was homozygous rather than heterozygous at the Aast9 locus (Table 2).

As we were only able to test a fraction of the signal crayfish populations in the UK (see James et al. 2014 for detailed distribution information) for *A. astaci,* comprehensively assessing the risk this pathogen poses to native crayfish in the UK was beyond the scope of the current study. Nevertheless, we located 10 native crayfish populations (confirmed extant at some time point between 2009 and 2014) within 15 km of an *A. astaci* infected signal crayfish population (Table 3). Of these, the population in River Cilcenni, South Wales, was closest (within 7.5 km) to infected signal crayfish (Table 3). These infected crayfish from the

Bachowey River were also within 15 km of an additional six extant native crayfish populations (Table 3). Due to the low spatial resolution of the river network data available it was, however, often not possible to determine if the waterbodies harbouring these native and invasive crayfish populations were connected.

Discussion

Using molecular diagnostics, we provide the first comprehensive study of *Aphanomyces astaci* prevalence in invasive signal crayfish populations from England and Wales. Whilst this affirms the perceived role of *A. astaci* causing native crayfish declines (Holdich, 2003), not all signal crayfish populations tested appeared to be infected. In fact, *A. astaci* was only detected in just over half (57%) of the tested UK signal crayfish populations, and within these populations the prevalence varied between 3 and 80%. While we cannot definitively declare those populations where we did not detect *A. astaci* as uninfected, our data show that, among signal crayfish populations, pathogen prevalence varies widely. Our findings contradict the traditional assumption that all American crayfish are carriers of *A. astaci* (see Cerenius et al. 2003) but are in agreement with other DNA-based studies focusing on distribution and prevalence of this pathogen. Recently, populations of American crayfish, in which *A. astaci* had not been detected, were reported in other European countries (Kozubíková et al. 2009; Skov et al. 2011; Filipová et al. 2013; Schrimpf et al. 2013; Tilmans et al. 2014). The situation in the UK seems to almost mirror that reported from France, with 53% (24 out of 45) of signal crayfish populations being infected with *A. astaci* and the pathogen prevalence ranging from 8 to 80% (Filipová et al. 2013).

In the current study, microsatellite genotyping revealed the presence of an *A. astaci*-positive DNA isolate congruent with the reference genotype group B strain (Grandjean et al. 2014) at eight of the nine loci tested. Such intra-genotype group variation has been reported

previously (Grandjean et al. 2014; Mrugała et al. 2016) therefore, it is likely that the DNA isolate from the UK belongs to genotype group B. This is perhaps unsurprising given that, within Europe, group B strains of *A. astaci* were first isolated from invasive signal crayfish in Sweden (Huang et al. 1995), which is considered as the country of origin for most signal crayfish introduced into the UK during the 1970s and 80s (Holdich et al. 1999). Isolation of this highly virulent strain of *A. astaci* (see Makkonen et al. 2012) may explain the mass mortalities of native white clawed crayfish in the UK following the introduction of signal crayfish (e.g. James et al. 2014). Although chronic *A. astaci* infections have been observed in other white clawed crayfish and other native European crayfish (e.g. Jussila et al. 2011; Kokko et al. 2012; Pârvulescu et al. 2012; Schrimpf et al. 2012; Kušar et al. 2013; Maguire et al. 2016), these may be caused by the less virulent strains from the "old" genotype group, A (Makkonen et al. 2012). The ability to identify *A. astaci* strains and their virulence would help inform risk assessment for native crayfish populations in the future, although better characterisation of all *A. astaci* genotypes is required before this can be exploited fully.

Given that the long-term conservation of native crayfish in the UK is generally considered to be dependent upon the translocation of animals into "Ark Sites" (Peay, 2009), and that resources for implementing such measures are limited, targeting removal of native crayfish populations at the greatest risk of extirpation is critical. Native European crayfish can co-exist with American crayfish for extended periods of up to 30 years in the absence of *A. astaci* (see Schulz et al. 2006; Dunn et al. 2009; Schrimpf et al. 2013; Skov et al. 2011; Westman et al. 2002), but are often rapidly extirpated if this pathogen is present (e.g. Holdich and Reeve, 1991; Vennerström et al. 1998; Bohman et al. 2006; Kozubíková et al. 2008). Therefore, native white clawed crayfish populations in close vicinity to *A. astaci*-infected signal crayfish are predicted to be at greater risk of local extinction than those neighbouring uninfected signal crayfish. Considering that only a portion of the signal crayfish populations

existing in the UK were screened in the current study, and of these only around half were infected with *A. astaci,* increased testing for this pathogen is needed to comprehensively assess native crayfish populations at greatest risk of disease transmission. Nevertheless, for the 13 signal crayfish populations where we detected *A. astaci* we identified one white clawed crayfish population recorded since 2009, located within 7.5 km. This native crayfish population inhabits the Cilcenni within the Wye catchment, South Wales, and was most recently detected in 2009. Given its proximity to infected signal crayfish, we recommend that translocating a subset of individuals from this population into an "Ark Site" is considered as a priority, although we acknowledge that increased screening of signal crayfish for *A. astaci* may reveal other native crayfish populations at greater risk of extirpation. Determining the exact order of translocation priority for the 10 native crayfish populations within 15 km of an *A. astaci* infected signal crayfish population is beyond the scope of the current study. For extant populations, factors that should be considered when assessing translocation priority include: proximity to infected crayfish, connectivity of water bodies housing native and infected invasive crayfish (particularly considering the ability of the pathogen not only to be transmitted via spores in the water but potentially also with fish; Oidtmann et al. 2002, Svoboda et al. 2016), prevalence of *A. astaci* in the nearest infected crayfish population, density of crayfish present in the native crayfish and neighbouring infected signal crayfish population, and whether any barriers in the environment exist that may prevent animals from either population dispersing. Additionally, as native crayfish populations can be rapidly extirpated by crayfish plague, surveying to confirm the persistence of populations under consideration for translocation should always be a pre-requisite.

Within the UK, this is currently the only comprehensive study that uses molecular methods to confirm the presence, and determine the prevalence of, *A. astaci* in invasive signal crayfish populations. The current study also provides the first record of *A. astaci*

genotype group B (known to contain virulent strains) from signal crayfish in the UK. *A. astaci* presence and prevalence, however, varied between populations. Although we cannot definitively declare those signal crayfish populations where we did not detect the *A. astaci* as uninfected, our findings show that pathogen prevalence can vary from very low to very high. Therefore, from a conservation perspective, the risk posed to native crayfish from different invasive crayfish populations may be asymmetric. As such, considering *A. astaci* prevalence data will improve risk assessments for native crayfish populations. Based on our findings we recommend increased *A. astaci* screening, using appropriate pathogen specific molecular methods, of non-native crayfish populations in the UK, to fully assess the risks to native crayfish and target populations for translocation. As part of this, those populations where we detected trace levels (i.e. below the limit of detection) of *A. astaci* should be re-tested to ascertain whether they are harbouring a low prevalence infection.

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Table 1: Prevalence (95% CI) and infection intensity of *Aphanomyces astaci* in 23 populations of invasive signal crayfish (*Pacifastacus leniusculus*) from the UK, where infection intensities are reported as semi-quantitative agent levels (Vrålstad et al. 2009): uninfected (A0-A1) and infected (A2-A5).

Population (location)	Catchment	Lat/Long (approx.)	Prevalence % (95% CI)	No. animals tested (n)	Agent level (range)
Wales					
*Sirhowy River (Caerphilly)	Usk	51°39′27.55″N, 003°11′22.95″W	0 (0-17)	30	A0
*Dderw farm pond (Powys)	N/A	52°01′45.07″N, 003°15′28.26″W	0 (0-17)	30	A0-A1
*Bachowey River 1 (Powys)	Wye	52°06′12.07″N, 003°12′58.09″W	50 (27-73)	20	A0-A3
Bachowey River 2 (Powys)	Wye	52°06′34.17″N, 003°13′51.26″W	23 (10-42)	30	A0-A3
*Gavenny River (Monmouthshire)	Usk	51°50′27.68″N, 003°00′10.56″W	47 (28-66)	30	A0-A3
*Mochdre Brook (Powys)	Severn	52°30′13.71″N, 003°20′52.70″W	75 (51-91)	20	A0-A4
England					
Broadmead Brook (Wiltshire)	Bristol Avon	51°29′23.51″N, 002°15′27.95″W	0 (0-17)	30	A0
St Catherine's Brook (South Gloucestershire)	Bristol Avon	51°25′59.13″N, 002°18′33.23″W	0 (0-17)	30	A0
Sutton Bingham Reservoir (Somerset)	N/A	50°54′01.26″N, 002°38′03.04″W	0 (0-17)	30	A0
River Wharfe 1 (North Yorkshire)	Yorkshire Ouse	54°06′08.94″N, 002°02′06.50″W	0 (0-17)	30	A0
River Riddle (Cumbria)	Derwent	54°15′03.43″N, 002°37′34.15″W	0 (0-17)	30	A0
Fenny Beck (West Yorkshire)	Yorkshire Ouse	53°39′00.42″N, 001°44′17.65″W	0 (0-17)	29	A0
Great Ouse (Suffolk)	Great Ouse	52°20′09.81″N, 000°31′49.58″E	0 (0-17)	30	A0
*River Lugg (Herefordshire)	Wye	52°10′00.78″N, 002°42′01.02″W	0 (0-17)	30	A0-A1
Tetbury Avon (Wiltshire)	Bristol Avon	51°35′30.10″N, 002°06′45.17″W	3 (0-17)	30	A0-A3
River Hamps (Staffordshire)	Trent	53°04′52.99″N, 001°54′26.65″W	20 (3-56)	10	A0-A3
River Wharfe 2 (North Yorkshire)	Yorkshire Ouse	54°03′56.31″N, 002°00′05.37″W	38 (9-76)	8	A0-A3
River Evenlode (Oxfordshire)	Thames	51°48′08.02″N, 001°21′53.10″W	28 (12-46)	29	A0-A3
River Thame (Aylesbury)	Thames	51°45′14.43″N, 001°01′14.28″W	80 (58-90)	30	A0-A3
River Wid (Norfolk)	Thames	52°33′41.50″N, 000°27′22.84″E	19 (6-38)	27	A0-A3
River Ash (Hertfordshire)	Thames	51°48′33.52″N, 000°00′22.04″E	7 (1-22)	30	A0-A3
River Lea (Hertfordshire)	Thames	51°47′59.58″N, 000°04′31.86″W	17 (6-35)	30	A0-A4
Bently Brook (Derbyshire)	Trent	53°01′54.10″N, 001°44′15.07″W	10 (2-27)	30	A0-A5

*Animals were processed in Charles University (Prague).

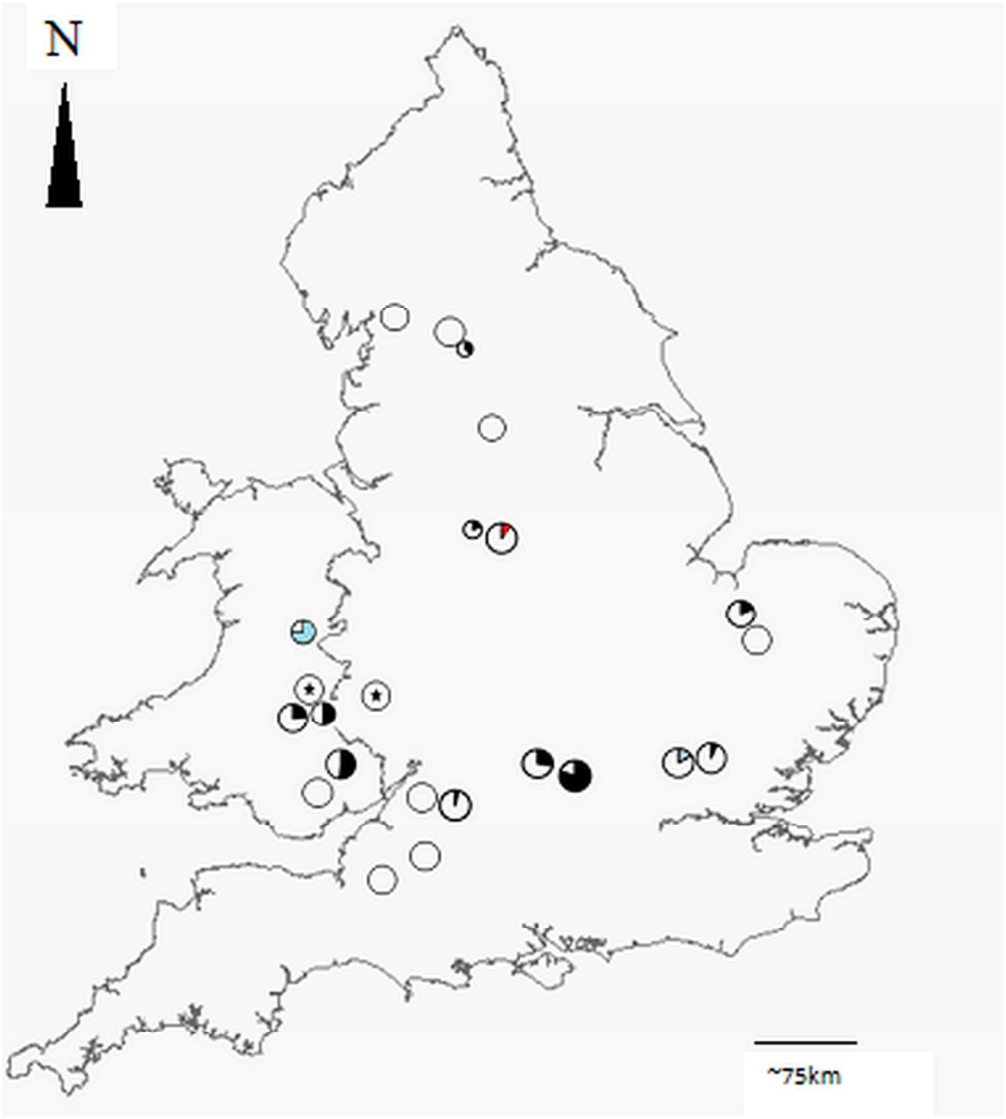
Table 2. Comparison of allele sizes of nine microsatellite loci from the reference strains of the *Aphanomyces astaci* genotype group B (Grandjean et al. 2014) and an *A. astaci*-positive signal crayfish (*Pacifastacus leniusculus*) from a UK population (Mochdre Brook).

Locus	Reference sequence (VI 03555)	UK population
Aast 2	142	142
Aast 4	87	87
Aast 6	148	148
Aast 7	215	215
Aast 9	164/182	164
Aast 10	132	132
Aast 12	226/240	226/240
Aast 13	202	202
Aast 14	248	248

Table 3. Location and year of the most recent record of native white clawed crayfish (*Austropotamobius pallipes*) populations (data from CrayBase: James et al. 2014) in close vicinity to an *Aphanomyces astaci* infected invasive signal crayfish (*Pacifastacus leniusculus*) population.

White clawed crayfish			Signal crayfish	
Population	**Location (country)**	**Most recent record**	**Population(s)**	**Proximity (km)**
Cilcenni	Wales	2009	Bachowey River 1 and 2	7.5
Scithwen	Wales	2014	Bachowey River 1 and 2	10.5
Clettwr	Wales	2014	Bachowey River 1 and 2	10.5
Rhiwiau Brook	Wales	2009	Bachowey River 1 and 2	12
Llynfi Dulas	Wales	2014	Bachowey River 1 and 2	15
River Ennig	Wales	2011	Bachowey River 1 and 2	15
Cwm Sheppard	Wales	2010	Bachowey River 1 and 2	15
Nant Onnau Fach	Wales	2010	Gavenny River	10.5
Lurscombe	England	2009	Tetbury Avon	15
Winterburn beck	England	2010	River Wharfe 2	10.5

Figure 1. Location of the invasive signal crayfish populations tested for *Aphanomyces astaci* in the current study using qPCR. For each population, the percentage of crayfish tested that were infected with *A. astaci* (i.e. the pathogen prevalence) is shown using a pie chart, with the shaded portion of each chart representing infected individuals, and the diameter of the circle the sample size (n=8-30). Black shading indicates that the highest infection intensity (reported as semi-quantitative agent levels, see Vrålstad et al. 2009) detected was A3, blue A4 and red A5. White circles show populations where the pathogen was not detected at any level (A0). Circles containing black stars represent those populations where trace levels of the pathogen (A1) were amplified. As an infection intensity of A1 is considered below the limit of detection for the method used (Vrålstad et al. 2009) these populations are classed as uninfected; although the possibility of them harbouring *A. astaci* at a low prevalence remains.



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