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Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA

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ABSTRACT

Aquatic invasive species (AIS) are important vectors for the introduction of novel pathogens which can, in turn, become drivers of rapid ecological and evolutionary change, compromising the persistence of native species. Conservation strategies rely on accurate information regarding presence and distribution of AIS and their associated pathogens to prevent or mitigate negative impacts, such as predation, displacement or competition with native species for food, space or breeding sites. Environmental DNA is increasingly used as a conservation tool for early detection and monitoring of AIS. We used a novel eDNA high-resolution melt curve (HRM) approach to simultaneously detect the UK endangered native crayfish (*Austropotamobius pallipes*), the highly invasive signal crayfish (*Pacifastacus leniusculus*) and their dominant pathogen, *Aphanomyces astaci* (causative agent of crayfish plague). We validated the approach using laboratory and field samples in areas with known presence or absence of both crayfish species as well as the pathogen, prior to the monitoring of areas where their presence was unknown. We identified the presence of infected signal crayfish further upstream than previously detected in an area where previous intensive eradication attempts had taken place, and the coexistence of both species in plague free catchments. We also detected the endangered native crayfish in an area where trapping had failed. With this method, we could estimate the distribution of native and invasive crayfish and their infection status in a rapid, cost effective and highly sensitive way, providing essential information for the development of conservation strategies in catchments with populations of endangered native crayfish.

1. Introduction

Invasive non-native species have become important drivers of global environmental change (Vitousek et al., 1996), although the importance of their impacts on biodiversity remains controversial (Russell and Blackburn, 2017). Their spread has been favoured by human-mediated activities (Crowl et al., 2008) in addition to natural dispersal, and, as a consequence have also become common vehicles for the introduction of novel pathogens (Randolph and Rogers, 2010). Invasive non-native species extend the geographic range of the pathogens they carry and facilitate host-switching (Peeler et al., 2011). In turn, pathogens play an important role in the evolution of communities but can also threaten the survival of native populations (Altizer et al., 2003). Co-introductions of parasites with non-native hosts are common; invasive species may bring novel infectious diseases that can infect native competitors, but can also act as hosts and effective dispersers for native diseases (Strauss et al., 2012). Invasive pathogens can have devastating effects on vulnerable native hosts, as their virulence tends to be higher than in the non-native species (Lymbery et al., 2014). Such pathogens seem particularly frequent in freshwater species, potentially reflecting the high susceptibility of freshwater ecosystems to non-native invasions (Moorhouse and Macdonald, 2015). Thus, early detection of both non-native hosts and parasites is critical for the control and management of the impacts caused by introduced diseases.

Detection of non-native species often occurs when populations have already established, spread from original source and altered the local environment (Vander Zanden et al., 2010; Zaiko et al., 2014). This is particularly the case in aquatic environments, where juveniles or larvae at the initial stages of introduction often have a patchy distribution, are difficult to identify using morphological techniques, and are easily missed by monitoring programmes (Pochon et al., 2013). Early detection is needed to make management actions such as eradication and control of invasive species more efficient and/or effective (Lodge et al., 2016) and as such is becoming fundamental for the management and control of aquatic

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invasive species (AIS; Vander Zanden et al., 2010). Analysis of environmental DNA (eDNA), *i.e.* free DNA molecules released from sources such as faeces, skin, urine, blood or secretions of organisms, is proving increasingly useful for detecting species that are difficult to identify and locate by more traditional and time-consuming methods (Biggs et al., 2015), such as endangered species (Dejean et al., 2011) and AIS at the early stages of their introduction (Bohmann et al., 2014; Dejean et al., 2012). Although still a relatively new tool, eDNA is becoming widely used for conservation (Biggs et al., 2015; Laramie et al., 2015; Spear et al., 2015; Thomsen and Willerslev, 2015) and protocols are being refined to increase its accuracy and reliability (Goldberg et al., 2016; Wilson et al., 2016). Quantitative PCR (qPCR) is commonly used to target particular species in eDNA samples (*e.g.* Ficetola et al., 2008; Thomsen et al., 2012) and, coupled with *in vitro* controls and amplicon sequencing, has proved a reliable method for the detection of invasive and endangered aquatic species (Klymus et al., 2015; Spear et al., 2015). In addition, qPCR is widely used to detect infectious agents in environmental samples (Guy et al., 2003), and can be particularly useful for the early detection of aquatic pathogens which can be introduced simultaneously with non-native species (Ganoza et al., 2006; Strand et al., 2014). High Resolution Melting (HRM) analysis is a qPCR-based method which facilitates identification of small variations in nucleic acid sequences by differences in the melting temperature of double stranded DNA depending on fragment length and sequence composition (Ririe et al., 1997). Analysis of HRM curves has been widely used for SNP genotyping as a fast method to discriminate species (Yang et al., 2009), including natives and invasives (Ramón-Laca et al., 2014). HRM has the potential for being used in AIS identification, including aquatic invasive pathogens, but it has not yet been applied to their detection from eDNA samples. We have used this method to investigate the distribution of the invasive signal crayfish (*Pacifastacus leniusculus*), carrier of the crayfish plague agent (*Aphanomyces astaci*) which is highly infective for native species (*e.g. Austropotamobius pallipes*), and the potential coexistence between native and invasive crayfish in UK populations.

Invasive non-native crayfish have been globally introduced, mainly for human consumption, and are known to seriously impact native ecosystems through predation, competition, disease transmission and hybridisation (*e.g.* Lodge et al., 2012). In Europe, non-indigenous crayfish mostly of North American origin have outnumbered their native counterparts in much of their range and represent one of the main threats to their persistence (Holdich et al., 2009). The distribution and abundance of native European crayfish species has been strongly influenced by high mortality rates associated with contracting crayfish plague (Schrimpf et al., 2012) through the introduction of North American freshwater crayfish around 1850 (Alderman, 1996). *P. leniusculus* was one of the first non-native species introduced to Europe and in the UK is displacing the native crayfish (*A. pallipes*) which has been classified as endangered in the UK (IUCN, 2017). Its success has been attributed to preadaptation, niche plasticity, the aggressive nature of the species (Chapple et al., 2012; Pintor et al., 2008) and/or the competitive advantage provided by the crayfish plague (Bubb et al., 2005; Dunn et al., 2009; Edgerton et al., 2004; Griffiths et al., 2004).

By using a novel approach to simultaneously identify both AIS and their major associated pathogens, we analysed the distribution of the highly invasive signal crayfish (*P. leniusculus*), the native crayfish (*A. pallipes*) and the crayfish plague pathogen (*A. astaci*) in areas where the presence of the signal crayfish is severely impacting the native populations, to identify potential areas of coexistence and refugia for the native species. We expected to find coexisting populations of both species more likely in locations where the crayfish plague has been historically and continually absent.

2. Materials and methods

2.1. Ex situ optimisation of eDNA methods

In order to optimise eDNA protocols an *ex-situ* pilot experiment was conducted by placing individual *P. leniusculus* in three isolated tanks, each with 2 L of water. After 24 h, they were removed and two 15 ml water samples were taken from each tank. The sampling was repeated 24 and 48 h after removal. Two ultrapure water blanks and four tank blanks (with no crayfish in) were also taken as controls during each sampling period. Immediately after collection, a standard method of preserving and extracting eDNA was applied by the addition of 33 ml of absolute ethanol and 1.5 ml of 3 M sodium acetate to samples and subsequent storage at −20 °C for a minimum of 24 h before DNA extraction (Ficetola et al., 2008). To recover precipitated DNA, samples were centrifuged to create a DNA pellet. The supernatant was discarded and the remaining pellet was air-dried before being subjected to DNA extraction. Extraction blanks consisting of ultrapure water in place of sampled water and tank blanks were used to test for any cross-contamination of the samples. Similarly, nine 15 ml water samples were taken, along with a system blank, at a local hatchery containing a population of *A. pallipes*, to test detection levels of native crayfish in aqueous eDNA samples.

2.2. Study populations and eDNA sample collection

We sampled six locations in the River Wye catchment and seven additional sites in the River Taff catchment, both in Wales, UK (Fig. 1a–c), as well as a total of 29 sites in two catchments from Southern England, the Itchen and Medway rivers (Fig. 1c; Table 1), all of them introduced c.1970. Records of the introduction of signal crayfish in Europe are very limited, but some evidence suggests that between 1976 and 1978 around 150,000 juvenile signal crayfish were introduced into Britain and other European countries from a hatchery in Simontorp, Sweden, which originally imported them from Lake Tahoe in California and Nevada, USA, in 1969 (Holdich and Lowery, 1988). After the Simontorp introductions, crayfish began to be imported directly from different American hatcheries (Holdich and Lowery, 1988), suggesting that the current populations could have different origins, and potentially initial infection status.

Welsh locations were selected based upon data from CrayBase (James et al., 2014a); two of the locations supported *A. pallipes* populations, with no evidence of *P. leniusculus* presence, three locations only had populations of *P. leniusculus* and the remaining eight locations could potentially have both *P. leniusculus* and *A. pallipes* or neither species, but their status was uncertain as these had not been previously monitored. Two out of the three *P. leniusculus* confirmed sites were known to contain *A. astaci* infected crayfish (James et al., 2017).

In the river Medway, *P. leniusculus* was thought to inhabit the upper catchment but the crayfish status downstream was unknown, while in the river Itchen *A. pallipes* was assumed to be present throughout most of the upper catchment and *P. leniusculus* had been recorded in few sites both upstream and downstream of *A. pallipes* presence (Rushbrook, 2014); Table 1). The infection status of both the Medway and Itchen crayfish populations was unknown.

Each site was subdivided into three sampling sites (upstream, midstream and downstream), separated where possible by *ca.* 500 m, to increase the area sampled. Between three and nine 15 ml water samples were taken from each sampling site simultaneously. All samples were collected *ca.* 1 m beneath the surface for ponds and in shallow areas of low flow streams and preserved as for the *ex-situ* experiment. Negative controls consisting of ultrapure water in place of river/pond water were taken before and after sampling, at each sampling site. Temperature, weather conditions, amount of shade cover, flow rate and pH were measured at each site (Table 1). Footwear was washed with Virkon™ and equipment disinfected with bleach between samplings to prevent the possible spread of *A. astaci* spores and DNA contamination between sites. All Wye sites which indicated presence of either crayfish species based on initial qPCR results were re-sampled the following year to assess reproducibility of positive amplifications at the sites (Table 1). To estimate the current presence of both host species, 25 standard