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Survey of the crayfish plague pathogen presence in the Netherlands reveals a new *Aphanomyces astaci* carrier

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ABSTRACT

North American crayfish species as hosts for the crayfish plague pathogen *Aphanomyces astaci* contribute to the decline of native European crayfish populations. At least six American crayfish species have been reported in the Netherlands but the presence of this pathogenic oomycete with substantial conservational impact has not yet been confirmed in the country. We evaluated *A. astaci* prevalence in Dutch populations of six alien crustaceans using species-specific quantitative PCR. These included three confirmed crayfish carriers (*Orconectes limosus, Pacifastacus leniusculus, Procambarus clarkii*), two recently introduced but yet unstudied crayfish (*Orconectes* cf. *virilis, Procambarus* cf. *acutus*), and a catadromous crab *Eriocheir sinensis*. Moderate levels of infection were observed in some populations of *O. limosus* and *P. leniusculus*. Positive results were also obtained for *E. sinensis* and two Dutch populations of *O.* cf. *virilis*. English population of the latter species was also found infected, confirming this taxon as another *A. astaci* carrier in European waters. In contrast, Dutch *P. clarkii* seem only sporadically infected, and the pathogen was not yet detected in *P.* cf. *acutus*. Our study is the first confirmation of crayfish plague infections in the Netherlands and demonstrates substantial variation in *A. astaci* prevalence among potential hosts within a single region, a pattern possibly linked to their introduction history and coexistence.

1. Introduction

The oomycete *Aphanomyces astaci* Schikora is the causative agent of the crayfish plague, a disease responsible for high mortalities of indigenous crayfish species throughout Europe (e.g., Alderman, 1996). It was suspected as early as in the 1960s that non-indigenous crayfish species (NICS) play a crucial role in the transmission of the crayfish plague pathogen to populations of native European crayfish (Unestam, 1969). All three North American crayfish invaders widely established in Europe, *Orconectes limosus* (Rafinesque), *Pacifastacus leniusculus* (Dana), and *Procambarus clarkii* (Girard), are confirmed carriers of *A. astaci* (Diéguez-Uribeondo and Söderhäll, 1993; Unestam, 1972; Vey et al., 1983). These species had been imported to Europe before 1975 for stocking purposes and have become widespread since then (Holdich et al., 2009; Kouba et al., 2014).

At least seven other crayfish species of North American and Australasian origin have become established in Europe more recently, mainly thanks to introductions from aquarium trade and aquaculture (Holdich et al., 2009). Five of these "new NICS" are of North American origin, and thus potential carriers of *A. astaci* (see Oidtmann, 2012; Unestam, 1972, 1969). However, it has been shown that the prevalence of *A. astaci* may substantially vary among species, regions, and even local populations (e.g., Filipová et al., 2013; Kozubíková et al., 2011a; Schrimpf et al., 2013a). Thus, the potential to spread *A. astaci* cannot be assessed unless a particular species (population) is tested for the presence of the pathogen. So far, only one of the new NICS, the calico crayfish *Orconectes immunis* (Hagen), has been confirmed as a vector of this pathogen (Filipová et al., 2013; Schrimpf et al., 2013b). Nevertheless, these findings highlight the potential of other newly introduced North American crayfish species to spread the crayfish plague agent.

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To date, seven non-indigenous crayfish species have been reported in the Netherlands (although the taxonomic status of some of them is not entirely clear; see Filipová et al., 2010, 2011). These include: narrow-clawed crayfish *Astacus leptodactylus* (first reported in 1982), spiny-cheek crayfish *Orconectes limosus* (1973), virile crayfish *O.* cf. *virilis* (2006), signal crayfish *Pacifastacus leniusculus* (2005), white river crayfish *P.* cf. *acutus* (2006), red swamp crayfish *Procambarus clarkii* (1989), and marbled crayfish *P. fallax* f. *virginalis* (2006) (Adema, 1989, 1982; Geelen, 1978, 1975; Geelen and Oomen, 1973; Soes and van Eekelen, 2006; Soes and Koese, 2010). While *A. leptodactylus* originates from Eastern Europe, the other six alien crayfish species found in the Netherlands are of North American origin. Although the present status of the marbled crayfish population is unclear (Soes and Koese, 2010), the country still harbors one of the highest numbers of potential crayfish plague carriers in Europe (see Kouba et al., 2014). Moreover, since the early 1930s Dutch waters have been invaded by the Chinese mitten crab *Eriocheir sinensis* (Herborg et al., 2003; Kamps, 1937), which can also get infected by the pathogen from carrier crayfish (Svoboda et al., 2014).

In contrast with apparently thriving alien crustaceans, Dutch populations of indigenous noble crayfish *Astacus astacus* have disappeared at an alarming rate since the second half of the twentieth century. Whereas during the period from 1660 to 1947, 38 Dutch localities were still inhabited by *A. astacus*, their number gradually decreased over time (Geelen, 1978), and presently only one residing population remains (Ottburg and Roessink, 2012).

The presence of *A. astaci* in the Netherlands was never officially confirmed although epizootics of crayfish plague were implicated as one of the major reasons for the decline of native crayfish in the Netherlands. For example, this disease was the presumed cause of the mass mortality of some of the last Dutch populations of *A. astacus* in the Roosendaalse Brook in 2001 (Niewold, 2002), since when only a single population in the Netherlands remains in an isolated pond near Arnhem. Infection by this pathogen has only been studied for one Dutch population of *A. leptodactylus* so far, and the few screened individuals tested negative (Roessink and Ottburg, 2012). As a reintroduction program aiming to increase the number of noble crayfish populations in the Netherlands has been recently launched (Ottburg and Roessink, 2012), knowledge on the distribution of the crayfish plague pathogen is of paramount importance for its success.

In the present study, we screened populations of all five well established North American alien crayfish species as well as one population of the Chinese mitten crab with the OIE-recommended (Oidtmann, 2012) molecular diagnostic methods to confirm the infection by *A. astaci*. Based on experience from other European countries, we expected a widespread presence of *A. astaci* in populations of the well-known and common *A. astaci* carriers (*O. limosus, P. leniusculus, P. clarkii*). We also hypothesized that individuals of *E. sinensis* would test positive, since they are in contact with North American crayfish in Dutch waters, and thus can get infected. For the first time, we also provide results of testing of two recently introduced crayfish taxa, *Orconectes* cf. *virilis* and *Procambarus* cf. *acutus*, for which no data on *A. astaci* infections were previously available. We assumed that due to their North American origin, they may also host *A. astaci* in European waters.

2. Materials and methods

2.1. Sampling and DNA extraction

To evaluate the presence of *A. astaci* in Dutch waters, populations of five North American alien crayfish (spiny-cheek crayfish *Orconectes limosus*, virile crayfish *Orconectes* cf. *virilis*, signal crayfish *Pacifastacus leniusculus*, white-river crayfish *Procambarus* cf. *acutus*, and red swamp crayfish *Procambarus clarkii*) and one Asian crab species that gets into contact with potential *A. astaci* carriers in Dutch freshwaters (Chinese mitten crab *Eriocheir sinensis*) were sampled. The approximate locations of the sampled populations are presented in Fig. 1. Their exact position, sampling details, and number of individuals sampled per population are summarized in Table 1.

Sample storage, processing and DNA isolation slightly differed as samples from the involved localities were processed independently in two laboratories. Selected samples of all five crayfish taxa were analyzed at the Central Veterinary Institute in Lelystad, the Netherlands (CVI). In parallel, other samples of four of these taxa (all but *P. leniusculus*) and samples of *E. sinensis* were analyzed at the Department of Ecology, Charles University in Prague, Czech Republic (CUNI). To confirm correct detection of the pathogen and to compare the quantitative results obtained in the two laboratories, a selection of DNA isolates was analyzed both at CUNI and at CVI.

Upon sampling, specimens were stored in plastic bottles filled with 96% ethanol (CUNI), or frozen and stored at −20 °C (CVI). We dissected either soft abdominal cuticle, any melanization on the body visible by naked eye, and pieces of two uropods (CUNI) or exclusively soft abdominal cuticle (CVI) from each crayfish individual. From crab specimens we used soft cuticle from telson and abdomen, 4 joints from chelipeds, second pair of maxillipeds, and any melanized wounds after a pereiopod loss. Dissection tools were cleaned with UV-light and sodium hydroxide, or with hydrogen peroxide and flame sterilization after dissection of each individual to prevent cross-contamination. The dissected tissues were pooled together in order to obtain one DNA isolate for each specimen.

Prior to DNA extraction, the tissues were mechanically disrupted and homogenized. Grinding in sterile mortars with liquid nitrogen was used at CUNI. At CVI, the tissues were homogenized with TeSeE PRECESS 24 homogenizer (BioRad) in IDEXX tissue dis-

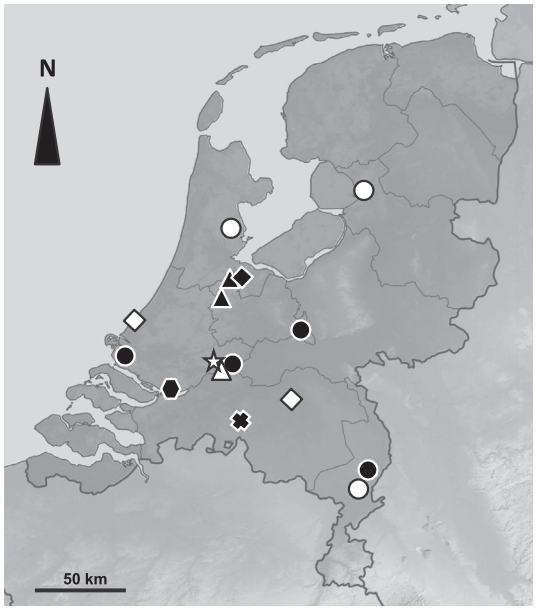

Fig. 1. Map of the Netherlands with approximate locations of analyzed populations of *O. limosus* (circle), *O.* cf. *virilis* (triangle), *P. leniusculus* (cross), *P. acutus* (star), *P. clarkii* (diamond) and *E. sinensis* (hexagon). Populations in which *A. astaci* infection was detected are indicated by black shapes, those without *A. astaci* detection by white shapes. In cases where sampled populations are in close vicinity to each other, only one location is marked in the map.

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