This is a “preproof” accepted article for *Parasitology*.
This version may be subject to change during the production process.

10.1017/S0031182025000022

Prevalence and diversity of *Aphanomyces astaci* in cambarid crayfish of Pennsylvania: Where native and introduced hosts meet

Adam Petrusek[1], Michaela Mojžišová[1], Adéla Mikešová[2], Radka Piálková[3], and David A. Lieb[4]

[1] Department of Ecology, Faculty of Science, Charles University, Prague, Czechia; [2] Department of Medical Biology, Faculty of Science, University of South Bohemia in České Budějovice, České Budějovice, Czechia; [3] Department of Zoology, Faculty of Science, University of South Bohemia in České Budějovice, České Budějovice, Czechia; [4] Pennsylvania Fish and Boat Commission, Bellefonte, PA, USA

Corresponding author: Adam Petrusek, Email: petrusek@natur.cuni.cz

Abstract

The crayfish plague pathogen *Aphanomyces astaci* (Oomycota: Saprolegniales) is native to North America but expanded with its crayfish hosts to other regions. In most of its invaded range, *A. astaci* haplotypes are associated with specific American crayfish, probably due to introduction bottlenecks, but haplotype diversity is higher and clear host-specific associations are lacking in its native range. However, little is known about the infection rate and load of this pathogen in North America. We investigated the distribution, prevalence, and genetic variation of *A. astaci* in Pennsylvania (eastern USA), where multiple native and introduced crayfish species (family Cambaridae) occur. We used *A. astaci*-specific quantitative PCR to screen 533 individuals representing eight crayfish species (two *Cambarus* and six *Faxonius*) from 49 sites. *Faxonius limosus*, an American species first introduced to Europe and carrier of *A. astaci* genotype group E, was of particular interest. We confirmed *A. astaci* infections in 76% of sites in all but one host taxon, with the pathogen infection rate and load comparable to established populations of North American crayfish studied in Europe and Japan. Despite the absence of highly infected hosts, we genotyped *A. astaci* from 14 sites. We only detected two mitochondrial haplotypes but nuclear markers indicated the presence of at least four distinct pathogen genotypes, none documented from invaded areas in Europe or Asia. Genotype group E was not detected in *F. limosus*, possibly due to limited spatial distribution of the original strain. Our results highlight both benefits and limitations of combining multiple pathogen genotyping methods.

Keywords: crayfish plague; native hosts; haplogroups; genotyping; host specificity

Introduction

The oomycete *Aphanomyces astaci* (Saprolegniales), a causative agent of crayfish plague, has been introduced from North America to several regions across the globe, where it threatens native crayfish populations or aquaculture production of species susceptible to this disease. Crayfish plague has a particularly strong impact in Europe, where it has been causing mass mortalities of local crayfish since the 19th century (Alderman, 1996; Holdich *et al.*, 2009), and in Japan where it has been implicated in the decline of the endemic Japanese crayfish *Cambaroides japonicus* (Martín-Torrijos *et al.*, 2018). Although the original mode of introduction of this pathogen to Europe remains unclear, most *A. astaci* strains documented in its invaded range were associated with introductions of particular non-native crayfish species of North American origin (Ungureanu *et al.*, 2020; Martín-Torrijos *et al.*, 2018). These crayfish usually serve as asymptomatic hosts of *A. astaci*, thanks to their long coevolutionary history with this pathogen.

Despite the coevolution, however, *A. astaci* is not a harmless commensal for its original hosts, as it rather behaves as an opportunistic pathogen. Its hyphae penetrating through the cuticle are stopped by an active response of the host's immune system and encapsulated by deposited melanin (Cerenius *et al.*, 2003), hosts from infected populations may show gross symptoms such as limb loss or visible melanised lesions on the body surface (e.g., Jussila *et al.*, 2016), and even otherwise non-symptomatic hosts may die with symptoms of acute crayfish plague when exposed to high doses of *A. astaci* spores or stressed (Diéguez-Uribeondo and Söderhäll 1993; Kozubíková *et al.*, 2011*a*; Aydin *et al.*, 2014). Furthermore, *A. astaci* may interact with other pathogens, resulting in further detrimental impacts to the hosts (Edsman *et al.*, 2015). Upon contact, North American crayfish infected with *A. astaci* may transfer it to species susceptible to crayfish plague (reviewed in Svoboda *et al.*, 2017). Outside the native range of both pathogen and hosts, cases of horizontal transfer of *A. astaci*

strains between different North American crayfish have also been occasionally documented (James *et al.*, 2017; Mojžišová *et al.*, 2022, 2024).

Most mass crayfish mortalities caused by *A. astaci* in Europe have been associated with four major pathogen genotype groups (labelled by capital letters A, B, D, and E; see Svoboda *et al.*, 2017; Ungureanu *et al.*, 2020 for review). Group A has been spreading through the continent since the 19th century without a specific original host. Groups B and D were introduced only after the mid-20th century with their highly invasive hosts, the signal crayfish *Pacifastacus leniusculus* and the red swamp crayfish *Procambarus clarkii*, respectively. These introductions likely happened repeatedly, as both host species were introduced to Europe for fisheries and aquaculture purposes in high numbers and multiple times (Henttonen and Huner 1999; Gherardi, 2006). The third widespread North American crayfish invader in Europe, the spiny-cheek crayfish *Faxonius limosus*, the original host of *A. astaci* group E (Kozubíková *et al.*, 2011*a*), however, has a different history, with apparently a single successful introduction in 1890 (Filipová *et al.*, 2011). Apart from the above-mentioned *A. astaci* genotype groups isolated to axenic cultures, additional strains of the pathogen introduced to Europe have been documented, either from mass mortalities or chronic infections of native crayfish (Grandjean *et al.*, 2014; Panteleit *et al.*, 2018; Mojžišová *et al.*, 2020), or from crayfish traded for ornamental purposes and subsequently released to the wild (Mojžišová *et al.*, 2024). The prevalence of *A. astaci* (i.e., the infection rate estimated by molecular detection methods) in its invasive host populations has been studied frequently in various countries where crayfish plague threatens native species (e.g., Kozubíková *et al.*, 2011*b*; Grandjean *et al.*, 2017; Mrugała *et al.*, 2017; Laffitte *et al.*, 2024). In contrast, much less attention has been paid to the distribution and diversity of *A. astaci* in its native range, North America, with no more than five studies available so far, four from the USA (Makkonen *et al.*, 2019; Panteleit *et al.*, 2019; Butler *et al.*, 2020; Martín-Torrijos *et al.*,

2021*b*) and one from Mexico (Martín-Torrijos *et al.*, 2023). A summary of the evidence from the USA (Martín-Torrijos *et al.*, 2021*b*) indicates, not surprisingly, a substantially higher diversity (i.e., presence of more mitochondrial haplotypes) of *A. astaci* in local crayfish hosts than across the Atlantic. The results also suggest that the association between particular host species and pathogen haplogroups in the USA is weaker, if present at all. However, the presence of matching *A. astaci* genotypes or haplogroups were confirmed in populations of *P. clarkii* and *P. leniusculus* from their source regions in the USA and from regions in Europe colonised by those species (Makkonen *et al.*, 2019; Martín-Torrijos *et al.*, 2021*b*). Data on the *A. astaci* infection rate in native North American crayfish populations are lacking altogether. Panteleit *et al.* (2019) applied quantitative PCR to screen for *A. astaci* in several US populations of the rusty crayfish *Faxonius rusticus*, but their study focused on the non-native range of this species, which is a widespread invader in North American waters (Durland Donahou *et al.*, 2024).

The aim of our study was to investigate the distribution, prevalence and diversity of *A. astaci* in Pennsylvania, a US state where multiple potential host species, both native to the region and introduced from other parts of the USA, come into contact (Lieb *et al.*, 2011*a*, 2011*b*). Among the native taxa, *F. limosus* deserves special attention as the first successfully established North American crayfish in Europe, with which *A. astaci* genotype group E was co-introduced (Kozubíková *et al.*, 2011*a*; Ungureanu *et al.*, 2020). The Delaware River basin, which covers eastern Pennsylvania, was assumed to be the source of the only successful introduction of *F. limosus* to Europe (Schikora, 1916; Henttonen and Huner, 1999), but phylogeographic data suggest that, at least for the Lower Delaware watershed, this is unlikely (Filipová *et al.*, 2011). The presence of *A. astaci* was confirmed in three non-native crayfish species in a small area of Pennsylvania (Lancaster County, Lower Susquehanna watershed) by Butler *et al.* (2020), and axenic cultures of a strain assigned by these authors to *A. astaci*

genotype group C were isolated from one of them, the Allegheny crayfish *Faxonius obscurus*.

We hypothesised that our broader screening in Pennsylvania would confirm a widespread presence of *A. astaci* in both introduced and native crayfish species, and that infection rates of this pathogen in host populations would vary substantially (corresponding to patterns documented from areas invaded by *A. astaci* hosts in other continents). For *A. astaci*-positive samples, we attempted to apply several complementary genotyping methods to differentiate between the pathogen strains involved. Considering the generally low infection loads usually observed in non-symptomatic *A. astaci* hosts (e.g., Tilmans *et al.*, 2014; Panteleit *et al.*, 2019; Mojžišová *et al.*, 2024), we expected that only a small fraction of such samples would allow for successful pathogen genotyping, but that we would nevertheless detect a substantial variation of the pathogen in our study region, with likely presence of yet uncharacterised *A. astaci* genotypes. Out of the already known ones, we expected to encounter *A. astaci* group E in *F. limosus*, and the genotype already reported by Butler *et al.* (2020) from *F. obscurus*. For the latter, we wanted to clarify its identity in respect to *A. astaci* group C, originally isolated from an entirely unrelated host (*P. leniusculus*) originating from the Pacific coast of British Columbia.

Materials and methods

Crayfish sampling

Crayfish were opportunistically sampled between 2017 and 2022 in central to eastern Pennsylvania (Fig. 1) from various lotic as well as lentic aquatic habitats, including small and large streams, rivers, ponds and lakes, as a part of a larger effort to survey the Susquehanna and Delaware River drainages of Pennsylvania for crayfish. Additional specimens were collected from streams in southeastern Pennsylvania during targeted efforts to document the

https://doi.org/10.1017/S0031182025000022

distribution of one of the state's rarest crayfish species, an undescribed member of the *Cambarus acuminatus* complex (Lieb *et al.*, 2008, 2011*a*, 2011*b*; Williams *et al.*, 2020). Crayfish from small streams (< 1 m wide), as well as from nearshore habitats of standing waters, were sampled with dip nets (41×38 cm) for ca 1-person hour; all available bottom substrates including root masses and aquatic vegetation were covered. In wider streams, crayfish were sampled using weighted seine nets stretched across the bottom, upstream of which the substrate was disturbed; 10 seine hauls were performed in ca 125-meter reach of each stream.

Crayfish were identified to species by morphological characteristics given in Swecker *et al.* (2010) and Thoma (2022). The number of processed crayfish specimens depended on local population density and capture success, and ranged from 1 to 30 per site and species. Altogether, 533 host individuals from 49 sites were analysed (Table 1, Figs. 1, S1), representing eight host crayfish taxa (for their common names, see Table 2): two certainly native to the region (*Cambarus bartonii*, *F. limosus*), two additional ones also likely native (*Cambarus robustus*, *Faxonius propinquus*), three certainly introduced (*Faxonius immunis*, *F. rusticus*, *F. virilis*), and one likely introduced to most, if not all sampled sites (*F. obscurus*). At 14 localities, coexisting crayfish species were collected (usually two, in one case three), in various combinations (Table 1).

Crayfish individuals were euthanised and preserved in 80% molecular-grade ethanol after sampling. Small specimens were kept whole, larger specimens were dissected before transport to the molecular laboratory of Charles University, Prague, Czechia. Only body parts suitable for screening for *A. astaci* (primarily soft abdominal cuticle and tail fan, i.e., uropods and telson, occasionally pereiopods) were used for DNA isolation.

Aphanomyces astaci *detection*

DNA from crayfish tissues was isolated following Oidtmann *et al.* (2006) and Kozubíková *et al.* (2008), by grinding cuticle cleaned from muscle and other soft tissues in liquid nitrogen (usually 40-60 mg, smaller amounts for very small specimens) and processing this homogenate with a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The isolation generally followed the manufacturer's protocol, the incubation time was at least 12 hours at 56 °C, and the elution to 100 µL of AE buffer pre-heated to 65 °C was performed twice to increase DNA yield (reaching the total DNA isolate volume of 200 µL). Two negative controls, consisting of tubes containing 50 µL of sterile water, were included with each isolation batch; one was kept open during mechanical tissue processing, the other while using the isolation kit.

The presence of *A. astaci* DNA was evaluated by quantitative real-time PCR (qPCR), using the TaqMan MGB-based protocol designed to specifically detect the internal transcribed spacer region (ITS) of *A. astaci*. For part of the samples, an assay from Vrålstad *et al.* (2009), following a slightly modified protocol given in Svoboda *et al.* (2014), was applied. The bulk of the samples, including part of the *A. astaci*-positive isolates previously tested with the Vrålstad *et al.* (2009) assay (see Table S1 for individual results), were analysed utilising the recently published assay of Strand *et al.* (2023), which has increased specificity over the original protocol. The reaction mix of 25 µL volume was based on TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Waltham, MA, USA) and contained 5 µL of the DNA isolate, primers (AphAstITS_15F, AphAstITS_145R; 500 nM) and TaqMan probe (AphAstITS_61T; 100 nM) according to the original protocol. The reactions were conducted in Bio-Rad iCycler iQ5 (Bio-Rad Laboratories, Hercules, CA, USA), using the EMM^2 thermal profile of Strand *et al.* (2023) with the annealing temperature of 58 °C. Data were processed using iQ5 2.1 Standard Edition Optical System Software (Bio-

Rad).

In each qPCR reaction, negative controls from the DNA isolation step were included, as well as no-template controls and four positive calibrants in duplicates (4-fold dilution series based on the synthetic oligonucleotide of the target ITS sequence, with starting concentration of 5×10^5 PCR-forming units (PFUs) per reaction). These were used to calculate the starting DNA quantities in the analysed samples. These were then assigned, according to Vrålstad *et al.* (2009), to semi-quantitative categories (agent levels), in which the amount of target DNA increases exponentially (see also footnote 4 of Table 1). Agent levels A0 (no target DNA detection) and A1 (traces of target DNA; less than 5 PFU in the reaction) were considered negative, agent levels from A2 (5 to 50 PFU) to A4 (10^3 to 10^4 PFU) were considered *A. astaci*-positive. Potential PCR inhibition was evaluated by screening a subset of randomly selected DNA isolates (including those yielding negative results) 10× diluted, and comparing the differences in cycle threshold (Ct) between non-diluted and diluted samples (Kozubíková *et al.*, 2011*b*).

For localities where at least five specimens of a given host species were collected, a 95% confidence interval of *A. astaci* prevalence, based on the detected infection rates, was calculated by the epi.conf function of epiR package v. 2.0.76 (Stevenson and Sergeant 2024), using R v. 4.3.3. (R Core Team 2024). Considering the inherent limitation of the molecular detection methods, especially when testing only a limited part of the host crayfish body, but also due to laboratory procedures (such as DNA extraction efficiency), the reported infection rate values including their confidence intervals have to be considered underestimations. However, this bias is analogous to other recent studies using a comparable methodology (e.g., Laffitte *et al.*, 2024; Mojžišová *et al.*, 2024).

Aphanomyces astaci *genotyping*

Three complementary genotyping approaches were used to assess the diversity of the crayfish plague pathogen in the studied hosts, as in Mojžišová *et al.* (2022, 2024). The methods target different markers in the *A. astaci* genome and characterise them by different methodologies, but each of them has been repeatedly successfully applied for mixed genome samples containing DNA of crayfish hosts as well as *A. astaci*. Specifically, we 1) sequenced fragments of two mitochondrial genes (following Makkonen *et al.,* 2018); 2) screened by qPCR for presence of five different anonymous nuclear markers (following Di Domenico *et al.,* 2021); and 3) attempted to characterise variation at nine polymorphic microsatellite loci developed for *A. astaci* (following Grandjean *et al.,* 2014). All these methods were originally validated on axenic cultures of strains representing different *A. astaci* genotype groups but their subsequent application on additional material (DNA isolated from axenic cultures or infected crayfish) revealed that the methods are also useful for characterisation of other *A. astaci* genotypes, in which they show marker combinations that differ from those of the reference laboratory strains (Grandjean *et al.,* 2014; Panteleit *et al.,* 2018, 2019; Di Domenico *et al.,* 2021; Mojžišová *et al.,* 2024).

For genotyping purposes, *A. astaci*-positive DNA isolates with agent level A3 (mid-range) or higher were selected. In cases when some genotyping method(s) yielded at least some results but others did not, DNA concentration in the respective isolates was further increased by precipitation with GlycoBlue Coprecipitant (Thermo Fisher Scientific, Waltham, MA, USA), and resuspension of the resulting pellet in a final volume of 20 to 50 µL.

First, we attempted to amplify fragments of mitochondrial genes for small and large subunits of ribosomal RNA (rnnS and rnnL) according to Makkonen *et al.* (2018), which were Sanger-sequenced in both directions at the DNA sequencing facility of the Faculty of

Science, Charles University, Prague. Obtained sequences were then compared with various *A. astaci* haplotypes documented from the areas invaded by this pathogen, specifically Europe and Japan (Makkonen *et al.*, 2018; Martín-Torrijos *et al.*, 2018) as well as from various parts of North America (Martín-Torrijos *et al.*, 2021*b*, 2023). The matching haplotypes are indicated by lower-case letters (e.g., "a").

In addition, we applied five qPCR assays developed by Di Domenico *et al.* (2021), which target anonymous genomic regions considered by Minardi *et al.* (2018) to be diagnostic for *A. astaci* genotype groups. Although more detailed analyses indicated that the identification of *A. astaci* genotypes by these assays is not unambiguous (Di Domenico *et al.*, 2021; Mojžišová *et al.*, 2024), combined information on mitochondrial haplogroup with results of this qPCR genotyping approach substantially increases the resolution compared to using either method alone. The successful amplification of a given assay is indicated by upper-case letters in the results (e.g., "B"), the combination of a particular mitochondrial haplotype and qPCR assay for a given sample is separated by slash (e.g., "a/C") throughout the text. The previously studied *A. astaci* genotype groups, originally characterised by random amplified polymorphic DNA (RAPD-PCR; Huang *et al.*, 1994; Diéguez-Uribeondo *et al.*, 1995; Kozubíková *et al.*, 2011*a*), have the following expected combinations of mtDNA/qPCR genotyping results (Makkonen *et al.*, 2018; Di Domenico *et al.*, 2021): group A: a/A; group B: b/B; group C: a/C; group D: d1/D or d2/D (two mtDNA haplotypes are known from Europe); group E: e/E.

Finally, for isolates with the highest concentrations of the pathogen DNA, we tried to characterise variation of nine microsatellite loci developed for *A. astaci* by Grandjean *et al.* (2014). The original protocol was generally followed, but each locus was amplified and analysed separately, considering that we expected unusual fragment lengths or atypical combinations of alleles. To check for consistency and ensure reproducibility, PCR and

fragment analyses were repeated multiple times for any sample and locus that deviated from the others or failed to amplify. In case the results were inconsistent, such loci were conservatively scored as ambiguous and were not considered when interpreting the results. The resulting multilocus genotypes (MLGs) were compared with the reference strains of known *A. astaci* genotype groups (Grandjean *et al.*, 2014, amended in Mojžišová *et al.*, 2020) as well with additional pathogen genotypes previously characterised by these microsatellite markers (Panteleit *et al.*, 2019; Mojžišová *et al.*, 2024).

Results

Distribution and prevalence of A. astaci

The presence of *Aphanomyces astaci* was detected at 37 sampling sites (76%; Table 1), in all but one crayfish host species (Table 2) and throughout the whole study area (Figs. 1, S1). The exception with no *A. astaci* detection was *Cambarus robustus*, of which only four individuals from three sites were analysed; in all the other host species studied, the pathogen was detected in the majority of their populations (Table 2, Fig. S1). Overall, *A. astaci* was reliably confirmed (with agent level A2 or higher) in 255 out of 533 individuals analysed (48%). When both *A. astaci* detection assays were applied on the same DNA isolates, the detection of the pathogen agreed in all but one sample of *Faxonius limosus*, which was very weakly positive with the Vrålstad *et al.* (2009) assay but not with the Strand *et al.* (2023) assay; and was conservatively scored as negative, with no influence on population-level results.

The proportion of individuals in which *A. astaci* was detected ranged from 0 to 100% per population, but the confidence intervals of prevalence were wide (Table 1). High *A. astaci* infection rates were found in populations of native hosts (such as the coexisting *C. bartonii* and *F. limosus* in Radley Run) as well as in introduced *F. virilis* or *F. rusticus*. There were a few host populations in which not even traces of *A. astaci* DNA (i.e., agent level A0)

were detected; in most of such cases, however, the number of analysed crayfish specimens were too low to conclude that the pathogen was scarce or even absent at those sites (Table 1). The only exception was the *F. virilis* population from Arrowmink Creek in the Delaware basin, from which 30 individuals were analysed without any *A. astaci* detection.

Pathogen genotyping

At least some *A. astaci* genotyping data was obtained from 24 DNA isolates representing five host species (Tables 2, 3), two native ones (*Cambarus bartonii*: 7 individuals, *F. limosus*: 4), two introduced ones (*Faxonius rusticus*: 6, *F. virilis*: 6) and one host species with uncertain status in the study area (*F. propinquus*: a single individual). For all but one of these samples, one of the five qPCR genotyping assays yielded positive results, and for all but two, partial sequences of at least one of the mitochondrial ribosomal genes were obtained (Tables 1, 3). The failures to obtain PCR products of the target markers were consistent despite multiple attempts. For ten isolates, microsatellite genotyping was at least partially successful (consistent scoring of at least 7 out of 9 analysed loci).

Most of the genotyped samples (19) yielded a combination of *A. astaci* mitochondrial haplotype a (as revealed by sequences of one of both ribosomal markers) and positive signal of qPCR assay C (a/C hereafter; Table 3; Fig. 2). Both DNA isolates for which mtDNA sequencing was unsuccessful were also positive for qPCR assay C. In one sample with haplotype a, no qPCR genotyping assay was positive. Two samples, however, stood out due to unusual combinations of mitochondrial haplotype and qPCR genotyping results. A single *A. astaci*-positive specimen of the native crayfish *C. bartonii* from Green Tree Run, Delaware basin, apparently carried an *A. astaci* genotype with mitochondrial haplotype a but positively reacting to qPCR assay B (a/B). Another unusual sample was that from *F. propinquus* collected in Abrahams Creek, Susquehanna basin, which yielded mitochondrial haplotype b

(as revealed by both ribosomal markers) but a positive signal from qPCR assay C (b/C).

The unique status of the two samples with a/B and b/C marker combinations was also supported by microsatellite genotyping (Tables 3, 4), which resulted in a combination of allele sizes previously not documented from any other *A. astaci* strains isolated to axenic cultures or genotyped directly from crayfish hosts. Microsatellite multilocus genotypes of DNA isolates that yielded haplotype a but qPCR assay C signal (a/C) indicate that these represent more distinct *A. astaci* strains differing at multiple microsatellite loci (Table 4). Three individuals of *C. bartonii* from two populations shared the same *A. astaci* MLG (PA3), other MLGs were represented by single DNA isolates. Three of them (PA3b, PA3c, PA3d), from non-native *F. virilis* and *F. rusticus*, shared allele combinations at most loci with PA3 from the native *C. bartonii* but differed in the extent of heterozygosity (Table 4). A MLG labelled 'rust2', from *F. rusticus* collected from North Branch Calkins Creek, Delaware basin, was very similar in allele composition to the *A. astaci* 'rust1-genotype' characterised from populations of the same species introduced in Wisconsin (Panteleit *et al.*, 2019), differing in allele composition at a single locus Aast9. While a single allele was consistently amplified from the Pennsylvania sample (suggesting a homozygosity at Aast9), the axenic isolates from Wisconsin were heterozygous (Table 4). Lastly, one DNA isolate from *C. bartonii* from Birch Run, Delaware basin, consistently yielded three peaks at the locus Aast6 (Table 4), likely due to mixed infection by more than one *A. astaci* strain (or co-infection with another related oomycete). Interestingly, the allele composition from this sample was otherwise similar to an *A. astaci* MLG recently characterised from established ornamental crayfish in Budapest, Hungary (Mojžišová *et al.*, 2024).

In two cases, *A. astaci* genotyping from different host species coexisting in the same streams was possible: from native *C. bartonii* and *F. limosus* in Bennets Run, Delaware basin, and from introduced *F. rusticus* and *F. virilis* in an unnamed tributary of Conodoguinet

Creek, Susquehanna basin. All those crayfish yielded the same a/C combination of mtDNA/qPCR markers, but microsatellite analysis was only successful for one of the two syntopic hosts (Table 3), so it was not possible to assess whether the two coexisting species shared the same *A. astaci* genotype.

Discussion

Our study, based on comparable methods to those repeatedly used to screen for *A. astaci* in the regions invaded by this pathogen, confirmed that both population-level prevalences and individual pathogen loads in cambarid crayfish in the USA are similar to those in Europe (see references below) and Japan (Mrugała *et al.*, 2017). Crayfish native as well as introduced to Pennsylvania carry *A. astaci* frequently; the pathogen was widespread in the study region, and except of one site (30 negative-testing non-native *Faxonius virilis* from Arrowmink Creek), we cannot claim with confidence that *A. astaci* prevalence in any population was low. The pattern that populations from nearby water bodies show contrasting infection rates is common (e.g., Laffitte *et al.*, 2024), and cambarid crayfish also tend to have relatively low infection loads (usually not exceeding agent level A4, i.e., moderate levels of *A. astaci* DNA) in their invaded ranges outside of North America (Tilmans *et al.*, 2014; Mrugała *et al.*, 2017; Mojžišová *et al.*, 2022, 2024; Laffitte *et al.*, 2024).

Strongly infected North American hosts with agent levels A5 and higher have only occasionally been reported in studies focusing on *A. astaci* screening. Such high infection loads, observed for example in *P. clarkii* in Brazil (Peiró *et al.*, 2016), *F. rusticus* in Wisconsin (Panteleit *et al.*, 2019), *P. clarkii* in France (Laffitte *et al.*, 2024) and *P. virginalis* in Hungary (Mojžišová *et al.*, 2024), are comparable to samples from susceptible crayfish hosts affected by acute crayfish plague (e.g., Vrålstad *et al.*, 2014; Caprioli *et al.*, 2018) and indicate extensive growth of the pathogen in host tissues. Such DNA isolates are particularly

suitable for *A. astaci* genotyping. In samples from Pennsylvania, we did not encounter any specimen with high agent levels. However, genotyping *A. astaci* from DNA isolates with agent level A4 (moderate; exceeding 1000 PFU in qPCR assay targeting the ITS marker) by the methods used in our study is usually successful (Grandjean *et al.*, 2014; Makkonen *et al.*, 2018; Di Domenico *et al.*, 2021). Even some samples with agent level A3 (below 1000 PFU) tend to yield positive results (especially when additional steps to increase DNA concentration in the isolates are applied; Mojžišová *et al.*, 2022), although microsatellite genotyping (Grandjean *et al.*, 2014) clearly requires higher concentration of pathogen DNA than either mitochondrial haplotyping (Makkonen *et al.*, 2018) or the qPCR genotyping assays (Di Domenico *et al.*, 2021).

Patterns of microsatellite variation from mixed genomic samples must be considered with caution, as not all loci characterised by Grandjean *et al.* (2014) are specific for *A. astaci* only, and coinfections by different strains of the pathogen (Mojžišová *et al.*, 2024) or by *A. astaci* and another oomycete may lead to unusual combination of detected allele sizes (as was also the case in one of our samples; Table 3). Furthermore, amplification of microsatellite loci from DNA isolates with low concentration of pathogen DNA may be inconsistent or fail entirely. However, when distinct microsatellite multilocus genotypes (MLGs) match other genotyping assays (as was the case in our MLGs PA1 and PA2 with b/C and a/B marker combination), or when corresponding alleles are observed in multiple host individuals (such as our MLG PA3), the results may be considered conclusive, and the use of these markers provides important additional insight into the pathogen variation.

Small differences characterised only by differing levels of heterozygosity, as observed between multilocus genotypes PA3 to PA3d, or between Pennsylvanian rust2 and reference genotype rust1 from *F. rusticus*, might possibly result from inconsistent amplification of low-concentration templates, so we cannot entirely rule out that these in fact represent the same *A.*

astaci genotypes. However, it should be noted that the variation of the respective loci was consistent across multiple polymerase chain reactions and fragment analyses, and such minor differences are comparable with already documented microsatellite MLG variation within *A. astaci* genotype groups (Grandjean *et al.*, 2014; Maguire *et al.*, 2016; James *et al.*, 2017; Mrugała *et al.*, 2017). Interestingly, Butler *et al.* (2020) also reported minor variation (in homo- vs. heterozygosity) in sequences of a gene for chitinase among four *A. astaci* strains isolated from different *F. obscurus* host specimens originating from the same population.

Our results confirm that studies focusing on diversity of *A. astaci* should combine, whenever possible, different available genotyping methods to provide more detailed insight into the patterns of variation of this pathogen. This is well illustrated when contrasting the results of mtDNA sequencing and qPCR genotyping based on nuclear markers. Each of these methods independently indicated the presence of at least two different *A. astaci* genotypes in our samples, and each of them pointed to the potential presence of *A. astaci* genotype group B, documented so far only from *P. leniusculus* in the western USA (Makkonen *et al.*, 2019) and from regions where this host species was introduced (Martín-Torrijos *et al.*, 2018; Ungureanu *et al.*, 2020). However, when the markers were evaluated together, we observed three distinct mtDNA/qPCR combinations (frequent a/C, and rare a/B and b/C), none of which matched *A. astaci* group B (with expected b/B pattern: Makkonen *et al.*, 2018; Di Domenico *et al.*, 2021). The distinctness of the two unusual *A. astaci* genotypes was also supported by microsatellite markers, which additionally indicated that the genotyped samples from Pennsylvania pooled under a/C comprised more strains.

Neither the two unique isolates with a/B and b/C genotyping combinations, nor the other samples characterised by microsatellite markers, corresponded to any *A. astaci* genotype known so far. The a/B marker combination has been documented from Europe, from the genotype “Up” causing mass mortalities of native crayfish in Czechia (Kozubíková-

Balcarová *et al.*, 2014; Mojžišová *et al.*, 2020) but also from a chronic infection of a narrow-clawed crayfish *Pontastacus leptodactylus* in the Danube (Panteleit *et al.*, 2018). Grandjean *et al.* (2014) speculated, based on allele composition, that this genotype may have been introduced to Europe with *P. leniusculus* (i.e., a host originating from the Pacific drainages of North America). The a/B sample from a cambarid host from Pennsylvania, *C. bartonii*, yielded distinctly different microsatellite MLG (Table 4), so it does not seem closely related to the "Up" genotype.

The b/C marker combination is new for *A. astaci*. Our genotyped sample from *F. propinquus* represents the first documented presence of *A. astaci* mitochondrial haplotype b in eastern North America (see Martín-Torrijos *et al.*, 2021*b*). Both qPCR genotyping and microsatellite MLG indicate that this is not any genotype known from *Pacifastacus*. Horizontal transmission of *A. astaci* genotype group B from this host to cambarids has been documented from the contact of invasive crayfish in Europe, either from signal crayfish (James *et al.*, 2017; Mojžišová *et al.*, 2022) or between various cambarids, some of which originated from ornamental aquaria (Mojžišová *et al.*, 2024). In none of those cases, however, the marker combination was close to that from Pennsylvanian *F. propinquus*. We assume that this *A. astaci* genotype may be natively occurring in cambarids. The unusual combination of mitochondrial haplotypes and nuclear markers raises a question about past evolution and dispersal of *A. astaci* strains, and potential gene flow between them, considering that sexual reproduction has not been documented from this pathogen (Diéguez-Uribeondo *et al.*, 2009; Rezinciuc *et al.*, 2015; Martínez-Ríos *et al.*, 2023). As Rezinciuc *et al.* (2015) speculated, it is possible that strains studied so far, in most cases isolated from *A. astaci*'s invaded range, represented the same mating type. It might be thus worth exploring whether the reproductive biology of this pathogen in its native range differs from that in Europe. It is noteworthy, however, that sexual structures are not known for most *Aphanomyces* species parasitising

animals (Diéguez-Uribeondo *et al.*, 2009).

The combination of mitochondrial and nuclear markers a/C, documented by us from multiple species, both native (*C. bartonii*, *F. limosus*) and invasive (*F. rusticus*, *F. virilis*) corresponds to that reported from *A. astaci* strains isolated from the locally non-native *F. obscurus* collected from one site in Pennsylvania by Butler *et al.* (2020). However, the same marker combination was also observed in *A. astaci* genotype group C (a strain isolated from *P. leniusculus* originating from British Columbia; Huang *et al.*, 1994), in another strain 'rust1' isolated from invasive populations of *F. rusticus* from Wisconsin (Panteleit *et al.*, 2019), and in an *A. astaci* genotype introduced, apparently with ornamental cambarid crayfish, to Budapest, Hungary (Mojžišová *et al.*, 2024). None of the Pennsylvania samples genotyped by microsatellites corresponded to genotype group C, as originally defined by Huang *et al.* (1994) by RAPD-PCR. One MLG characterised by us from *F. rusticus*, however, had an allele composition very similar, although not identical, to a genotype isolated from the same host elsewhere (Panteleit *et al.*, 2019), which may indicate that this invasive crayfish contributes to dispersal for several related *A. astaci* genotypes. However, our study indicates that even at a limited spatial scale, the strain variation of *A. astaci* within both native (*C. bartonii*) and invasive (*F. rusticus*) cambarid crayfish may be substantial.

We failed to amplify some of the diagnostic markers (mitochondrial ribosomal subunits or any of the nuclear markers targeted by qPCR assays) from several samples. Specifically, no qPCR assay was positive for one sample from infected *C. bartonii*. We do not know whether this represents an infection by a distinct *A. astaci* genotype that lacks all of the target regions in its genome or simply an amplification failure. Considering a relatively low amount of pathogen DNA in that sample, the latter explanation is possible although its mtDNA sequencing was successful. However, the existence of "non-amplifying" *A. astaci* strains is also very likely. In fact, we found it surprising that the assays based on anonymous

nuclear markers, primarily developed for fast screening for the common *A. astaci* genotypes causing mass mortalities in Europe (Di Domenico *et al.,* 2021), turned out to be usable in the native range of this pathogen, where substantially higher intraspecific variation even at a small regional scale would be expected (Martín-Torrijos *et al.,* 2021*b*).

An occasional repeated failure to amplify one of the mtDNA markers (attempted multiple times) could have been caused by previously undetected intraspecific variation in the primer region that was designed to avoid, as much as possible, amplification of other oomycetes (Makkonen *et al.,* 2018). Indeed, consistent failure to amplify the large mitochondrial ribosomal subunit was reported from *A. astaci* infecting *P. clarkii* hosts in Costa Rica (Martín-Torrijos *et al.,* 2021*a*). In two cases, when none of the mitochondrial markers amplified, and thus no sequence-based confirmation of the species identity was available, the observed patterns might also be explained by an undetected presence of another, closely related *Aphanomyces* species that cross-reacts with the ITS-based qPCR assay of Strand *et al.* (2023). This is not impossible, considering the limited knowledge of *Aphanomyces* spp. that occur on American crayfish (but see Kozubíková-Balcarová *et al.,* 2013; Makkonen *et al.,* 2019; Butler *et al.,* 2020) or in their environments, and also in the light of recent discovery of *A. fennicus*, a species closely related to *A. astaci* but apparently avirulent to crayfish (Viljamaa-Dirks and Heinikainen, 2019). *Aphanomyces fennicus* cross-reacted with a previously used detection assay of Vrålstad *et al.* (2009) but also with one of the nuclear qPCR genotyping assays of Di Domenico *et al.* (2021). A scenario of other cross-reacting species presence seems feasible in the case of *C. bartonii* from Bradly Run (sample PA-4, Table 3), less so for *F. rusticus* from the tributary of Yellow Breeches Creek (sample PA-326), for which seven microsatellite markers were scored, with allele sizes overlapping with the multilocus genotype SSR-PA3 repeatedly scored from *C. bartonii* (Table 4).

As pointed out above, genotyping *A. astaci* from mixed-genome samples, isolated directly from chronically infected hosts, is challenging due to low concentration of target DNA as well as potential cross-amplification of non-target taxa. To avoid methodological biases and to better characterise *A. astaci* diversity from its native range, DNA isolates from axenic cultures obtained from infected hosts (such as those from Panteleit *et al.*, 2019; Butler *et al.*, 2020; Martín-Torrijos *et al.*, 2021*a*, 2021*b*) should be studied in more detail, and by multiple genotyping methods. Furthermore, alternative variable markers to microsatellites (e.g., single nucleotide polymorphism arrays), optimised for genotyping from mixed genome samples with low *A. astaci* agent levels, could be developed. While the presently available microsatellite markers are convenient when genotyping the pathogen in laboratory cultures or in crayfish plague outbreaks of susceptible host species (e.g., Grandjean *et al.*, 2014; Vrålstad *et al.*, 2014; Kaldre *et al.*, 2017), the success is substantially lower for hosts that act as *A. astaci* asymptomatic carriers and tend to have much lower infection loads.

To conclude, our study confirmed that *A. astaci* infections are common across the study area and in most studied host crayfish species, regardless of their native or invasive status in Pennsylvania. We revealed several yet unknown *A. astaci* genotypes (characterised by multiple marker combinations). Some of the crayfish taxa apparently host more than one *A. astaci* genotype in the study region. Considering the number of host species and populations screened, however, the observed *A. astaci* variation, especially at the level of mitochondrial haplotypes, was lower than we expected. Specifically, we failed to find genotype group E introduced with *F. limosus* to Europe; all four infected specimens of this host species, from which *A. astaci* could be genotyped, carried another strain. Apparently, *A. astaci* group E, if present in Pennsylvania, is not particularly common in the region studied by us. It may have declined with *F. limosus*, a species that is a widespread invader in Europe (Kouba *et al.*, 2014) but has been replaced at many localities in Pennsylvania by introduced

crayfish species (Lieb *et al.*, 2011*b*). *A. astaci* group E could have also been locally displaced by some other, more successful strain (as recorded in coexisting non-native cambarid crayfish in Budapest; Mojžišová *et al.*, 2024), possibly even one introduced by some of the invasive crayfish taxa in the region. Most likely, however, this particular *A. astaci* genotype has a spatially restricted distribution and might eventually be found in other parts of *F. limosus* range. If distributed over a limited area only, the presence of this genotype may even indicate the potential source region for its host's introduction to Europe.

Supplementary material. The supplementary material for this article can be found at [DOI].

Acknowledgements. We thank West Liberty University Crayfish Lab and Jarren Uplinger, Emily Lieb, Allison Moll, and Sidney Shaw from the Pennsylvania Fish and Boat Commission's Natural Diversity Section for assistance in the field. Two anonymous referees provided feedback that improved clarity of the manuscript.

Author's contribution. AP, DL and MM planned the study. DL collected samples in the field together with his assistants and pre-processed them. MM, AM and RP performed laboratory analyses, MM and AP interpreted the data, and DL provided locally specific context. AP drafted the manuscript, with input from MM, DL and AM, and revised it based on referees' feedback. All authors approved the final version.

Financial support. Pennsylvania Department of Conservation & Natural Resources Wild Resource Conservation Program funded the crayfish collections through grant agreement WRCP-19601. Michaela Mojžišová was supported by a Charles University project SVV 260680.

Competing interests. The authors declare there are no conflicts of interest.

Ethical standards. Not applicable.

References

Alderman, DJ (1996) Geographical spread of bacterial and fungal diseases of crustaceans. *Revue scientifique et technique de l'Office international des épizooties*, **15**, 603–632. doi: 10.20506/rst.15.2.943

Aydin, H, Kokko, H, Makkonen, J, Kortet, R, Kukkonen, H and Jussila, J (2014) The signal crayfish is vulnerable to both the As and the PsI-isolates of the crayfish plague. *Knowledge and Management of Aquatic Ecosystems*, **413**, 03. doi: 10.1051/kmae/2014004

Butler, E, Crigler, P, Robbins, G and Blair, JE (2020) Preliminary survey of *Aphanomyces* sp. associated with native and invasive crayfish in the Lower Susquehanna watershed of South Central Pennsylvania. *Journal of Freshwater Ecology*, **35**, 223–233. doi: 10.1080/02705060.2020.1779141

Caprioli, R, Mrugała, A, Domenico, MD, Curini, V, Giansante, C, Cammà, C and Petrusek, A (2018) *Aphanomyces astaci* genotypes involved in recent crayfish plague outbreaks in central Italy. *Diseases of Aquatic Organisms*, **130**, 209–219. doi: 10.3354/dao03275

Cerenius, L, Bangyeekhun, E, Keyser, P, Söderhäll, I and Söderhäll, K (2003) Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*. *Cellular Microbiology*, **5**, 353-357. doi: 10.1046/j.1462-5822.2003.00282.x

Diéguez-Uribeondo, J and Söderhäll, K (1993) *Procambarus clarkii* Girard as a vector for the crayfish plague fungus, *Aphanomyces astaci* Schikora. A*quacutture and Fisheries Management*, **24**, 761–765. doi: 10.1111/j.1365-2109.1993.tb00655.x

Diéguez-Uribeondo, J, Huang, T, Cerenius, L and Söderhäll, K (1995) Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii*. *Mycological Research*, **99**, 574–578. doi: 10.1016/S0953-7562(09)80716-8

Diéguez-Uribeondo, J, García, MA, Cerenius, L, Kozubíková, E, Ballesteros, I, Windels, C, Weiland, J, Kator, H, Söderhäll, K and Martín, MP (2009) Phylogenetic relationships among plant and animal parasites and saprotrophs in *Aphanomyces* (Oomycetes). *Fungal Genetics and Biology*, **46**, 365–376. doi: 10.1016/j.fgb.2009.02.004

Di Domenico, M, Curini, V, Caprioli, R, Giansante, C, Mrugała, A, Mojžišová, M, Cammà, C and Petrusek, A (2021) Real-time PCR assays for rapid identification of common *Aphanomyces astaci* genotypes. *Frontiers in Ecology and Evolution*, **9**, 597585. doi: 10.3389/fevo.2021.597585

Durland Donahou, A, Conard, W, Dettloff, K, Fusaro, A and Sturtevant, R (2024) *Faxonius rusticus* (Girard, 1852): U.S. Geological Survey Nonindigenous Aquatic Species Database, Gainesville, FL. Revision Date: 19 January 2024. https://nas.er.usgs.gov/queries/FactSheet.aspx?speciesID=214 (accessed 23 August 2024)

Edsman, L, Nyström, P, Sandström, A, Stenberg, M, Kokko, H, Tiitinen, V, Makkonen, J and Jussila, J (2015) Eroded swimmeret syndrome in female crayfish *Pacifastacus leniusculus* associated with *Aphanomyces astaci* and *Fusarium* spp. infections. *Diseases of Aquatic Organisms*, **112**, 219-228. doi: 10.3354/dao02811

Filipová, L, Lieb, DA, Grandjean, F and Petrusek, A (2011) Haplotype variation in the spiny-cheek crayfish *Orconectes limosus*: Colonization of Europe and genetic diversity of native stocks. *Journal of the North American Benthological Society*, **30**, 871–881. doi: 10.1899/10-130.1

Gherardi, F (2006) Crayfish invading Europe: The case study of *Procambarus clarkii*. *Marine and Freshwater Behaviour and Physiology*, **39**, 175–191. doi: 10.1080/10236240600869702

Grandjean, F, Vrålstad, T, Diéguez-Uribeondo, J, Jelić, M, Mangombi, J, Delaunay, C, Filipová, L, Rezinciuc, S, Kozubíková-Balcarová, E, Guyonnet, D, Viljamaa-Dirks, S and Petrusek, A (2014) Microsatellite markers for direct genotyping of the crayfish plague pathogen *Aphanomyces astaci* (Oomycetes) from infected host tissues. *Veterinary Microbiology*, **170**, 317–324. doi: 10.1016/j.vetmic.2014.02.020

Grandjean, F, Roques, J, Delaunay, C, Petrusek, A, Becking, T and Collas, M (2017) Status of *Pacifastacus leniusculus* and its role in recent crayfish plague outbreaks in France: Improving distribution and crayfish plague infection patterns. *Aquatic Invasions*, **12**, 541–549. doi: 10.3391/ai.2017.12.4.10

Henttonen, P and Huner, JV (1999) The introduction of alien species of crayfish in Europe: A historical introduction. In Gherardi, F (ed.). *Crayfish in Europe as alien species: How to make the best of a bad situation? Crustacean Issues 11*. Rotterdam, The Netherlands: A.A. Balkema, pp. 13–22. doi: 10.1201/9781315140469-2

Holdich, DM, Reynolds, JD, Souty-Grosset, C and Sibley, PJ (2009) A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowledge and Management of Aquatic Ecosystems*, **394–395**, 11. doi: 10.1051/kmae/2009025

Huang, T, Cerenius, L and Söderhäll, K (1994) Analysis of genetic diversity in the crayfish plague fungus *Aphanomyces astaci* by random amplification of polymorphic

DNA. *Aquaculture*, **126**, 1–9. doi: 10.1016/0044-8486(94)90243-7

James, J, Mrugała, A, Oidtmann, B, Petrusek, A and Cable, J (2017) Apparent interspecific transmission of *Aphanomyces astaci* from invasive signal to virile crayfish in the UK. *Journal of Invertebrate Pathology*, **145**, 68–71. doi: 10.1016/j.jip.2017.02.003

Jussila, J, Tiitinen, V, Edsman, L, Kokko, H and Fotedar, R (2016) Signal crayfish in Lake Saimaa could be maladapted to the local conditions due to *Aphanomyces astaci* infection: a seven-year study. *Freshwater Crayfish*, **22**, 53–60. doi: 10.5869/fc.2016.v22-1.53

Kaldre, K, Paaver, T, Hurt, M and Grandjean, F (2017) First records of the non-indigenous signal crayfish (*Pacifastacus leniusculus*) and its threat to noble crayfish (*Astacus astacus*) populations in Estonia. *Biological Invasions*, **19**, 2771–2776. doi: 10.1007/s10530-017-1496-z

Kouba, A, Petrusek, A and Kozák, P (2014) Continental-wide distribution of crayfish species in Europe: Update and maps. *Knowledge and Management of Aquatic Ecosystems*, **413**, 05. doi: 10.1051/kmae/2014007

Kozubíková, E, Petrusek, A, Ďuriš, Z, Martín, MP, Diéguez-Uribeondo, J and Oidtmann, B (2008) The old menace is back: Recent crayfish plague outbreaks in the Czech Republic. *Aquaculture*, **274**, 208–217. doi: 10.1016/j.aquaculture.2007.11.015

Kozubíková, E, Viljamaa-Dirks, S, Heinikainen, S and Petrusek, A (2011*a*) Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague pathogen *Aphanomyces astaci*. *Journal of Invertebrate Pathology*, **108**, 214–216. doi: 10.1016/j.jip.2011.08.002

Kozubíková, E, Vrålstad, T, Filipová, L and Petrusek, A (2011*b*) Re-examination of the prevalence of *Aphanomyces astaci* in North American crayfish populations in Central Europe by TaqMan MGB real-time PCR. *Diseases of Aquatic Organisms*, **97**, 113–125.

doi: 10.3354/dao02411

Kozubíková-Balcarová, E, Koukol, O, Martín, MP, Svoboda, J, Petrusek, A and Diéguez-Uribeondo J (2013) The diversity of oomycetes on crayfish: Morphological vs. molecular identification of cultures obtained while isolating the crayfish plague pathogen. *Fungal Biology*, **117**, 682–691. doi: 10.1016/j.funbio.2013.07.005

Kozubíková-Balcarová, E, Beran, L, Ďuriš, Z, Fischer, D, Horká, I, Svobodová, J and Petrusek, A (2014) Status and recovery of indigenous crayfish populations after recent crayfish plague outbreaks in the Czech Republic. *Ethology Ecology & Evolution*, **26**, 299–319. doi: 10.1080/03949370.2014.897652

Laffitte, M, Mojžišová, M, Delaunay, C, Collas, M, Petrusek, A and Grandjean, F (2024) Prevalence of the crayfish plague pathogen in red swamp crayfish populations in western France: How serious is the risk for the native white-clawed crayfish? *Journal of Invertebrate Pathology*, **205**, 108128. doi: 10.1016/j.jip.2024.108128

Lieb, DA, Carline, RF, Rosenberger, JL and Mengel, VM (2008) The discovery and ecology of a member of the *Cambarus acuminatus* complex (Decapoda: Cambaridae) in Valley Creek, southeastern Pennsylvania. *Journal of Crustacean Biology*, **28**, 439–450. doi: 10.1651/07-2877R.1

Lieb, DA, Bouchard, RW and Carline, RF (2011*a*) Crayfish fauna of southeastern Pennsylvania: Distributions, ecology, and changes over the last century. *Journal of Crustacean Biology*, **31**, 166–178. doi: 10.1651/10-3287.1

Lieb, DA, Bouchard, RW, Carline, RF, Nuttall, TR, Wallace, JR and Burkholder, CL (2011*b*) Conservation and management of crayfishes: Lessons from Pennsylvania. *Fisheries*, **36**, 489–507. doi: 10.1080/03632415.2011

Maguire, I, Jelić, M, Klobučar, G, Delpy, M, Delaunay, C and Grandjean, F (2016) Prevalence of the pathogen *Aphanomyces astaci* in freshwater crayfish populations in

Croatia. *Diseases of Aquatic Organisms*, **118**, 45–53. doi: 10.3354/dao02955

Makkonen, J, Jussila, J, Panteleit, J, Keller, NS, Schrimpf, A, Theissinger, K, Kortet, R, Martín-Torrijos, L, Sandoval-Sierra, JV, Diéguez-Uribeondo, J and Kokko, H (2018) MtDNA allows the sensitive detection and haplotyping of the crayfish plague disease agent *Aphanomyces astaci* showing clues about its origin and migration. *Parasitology*, **145**, 1210–1218. doi: 10.1017/S0031182018000227

Makkonen, J, Kokko, H, Gökmen, G, Ward, J, Umek, J, Kortet, R, Petrusek, A and Jussila, J (2019) The signal crayfish (*Pacifastacus leniusculus*) in Lake Tahoe (USA) hosts multiple *Aphanomyces* species. *Journal of Invertebrate Pathology*, **166**, 107218. doi: 10.1016/j.jip.2019.107218

Martín-Torrijos, L, Kawai, T, Makkonen, J, Jussila, J, Kokko, H and Diéguez-Uribeondo, J (2018) Crayfish plague in Japan: A real threat to the endemic *Cambaroides japonicus*. *PLoS ONE*, **13**, e0195353. doi: 10.1371/journal.pone.0195353

Martín-Torrijos, L, Kokko, H, Makkonen, J, Jussila, J and Diéguez-Uribeondo, J (2019) Mapping 15 years of crayfish plague in the Iberian Peninsula: The impact of two invasive species on the endangered native crayfish. *PLoS One*, **14**, e0219223. doi: 10.1371/journal.pone.0219223

Martín-Torrijos, L, Correa-Villalona, A J, Azofeifa-Solano, J C, Villalobos-Rojas, F, Wehrtmann, I S and Diéguez-Uribeondo, J (2021*a*) First detection of the crayfish plague pathogen *Aphanomyces astaci* in Costa Rica: European mistakes should not be repeated. *Frontiers in Ecology and Evolution*, **9**, 623814. doi: 10.3389/fevo.2021.623814

Martín-Torrijos, L, Martínez-Ríos, M, Casabella-Herrero, G, Adams, SB, Jackson, CR and Diéguez-Uribeondo, J (2021*b*) Tracing the origin of the crayfish plague pathogen *Aphanomyces astaci* to the Southeastern United States. *Scientific Reports*, **11**, 9332. doi: 10.1038/s41598-021-88704-8

Martín-Torrijos, L, Hernández-Pérez, A, Monroy-López, JF and Diéguez-Uribeondo, J (2023) *Aphanomyces astaci* in Mexico: A new haplotype from dwarf crayfish *Cambarellus montezumae*. *Journal of Invertebrate Pathology*, **201**, 108000. doi: 10.1016/j.jip.2023.108000

Martínez-Ríos, M, Martín-Torrijos, L and Diéguez-Uribeondo, J (2023) Protocols for studying the crayfish plague pathogen, *Aphanomyces astaci*, and its host-pathogen interactions. *Journal of Invertebrate Pathology*, **201**, 108018. doi: 10.1016/j.jip.2023.108018

Minardi, D, Studholme, DJ, van der Giezen, M, Pretto, T and Oidtmann, B (2018) New genotyping method for the causative agent of crayfish plague (*Aphanomyces astaci*) based on whole genome data. *Journal of Invertebrate Pathology*, **156**, 6–13. doi: 10.1016/j.jip.2018.06.002

Mojžišová, M, Mrugała, A, Kozubíková-Balcarová, E, Vlach, P, Svobodová, J, Kouba, A and Petrusek, A (2020) Crayfish plague in Czechia: Outbreaks from novel sources and testing for chronic infections. *Journal of Invertebrate Pathology*, **173**, 107390. doi: 10.1016/j.jip.2020.107390

Mojžišová, M, Svobodová, J, Kozubíková-Balcarová, E, Štruncová, E, Stift, R, Bílý, M, Kouba, A and Petrusek, A (2022) Long-term changes in the prevalence of the crayfish plague pathogen and its genotyping in invasive crayfish species in Czechia. *NeoBiota*, **74**, 105–127. doi: 10.3897/neobiota.74.79087

Mojžišová, M, Weiperth, A, Gebauer, R, Laffitte, M, Patoka, J, Grandjean, F, Kouba, A and Petrusek, A (2024) Diversity and distribution of *Aphanomyces astaci* in a European hotspot of ornamental crayfish introductions. *Journal of Invertebrate Pathology*, **202**, 108040. doi: 10.1016/j.jip.2023.108040

Mrugała, A, Kawai, T, Kozubíková-Balcarová, E and Petrusek, A (2017) *Aphanomyces*

astaci presence in Japan: A threat to the endemic and endangered crayfish species *Cambaroides japonicus*? *Aquatic Conservation: Marine and Freshwater Ecosystems*, **27**, 103–114. doi: 10.1002/aqc.2674

Oidtmann, B, Geiger, S, Steinbauer, P, Culas, A and Hoffmann, R (2006) Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction. *Diseases of Aquatic Organisms*, **72**, 53–64. doi: 10.3354/dao072053

Panteleit, J, Keller, NS, Diéguez-Uribeondo, J, Makkonen, J, Martín-Torrijos, L, Patrulea, V, Pîrvu, M, Preda, C, Schrimpf, A and Pârvulescu, L (2018) Hidden sites in the distribution of the crayfish plague pathogen *Aphanomyces astaci* in Eastern Europe: Relicts of genetic groups from older outbreaks? *Journal of Invertebrate Pathology*, **157**, 117–124. doi: 10.1016/j.jip.2018.05.006

Panteleit, J, Horvath, T, Jussila, J, Makkonen, J, Perry, W, Schulz, R, Theissinger, K and Schrimpf, A (2019) Invasive rusty crayfish (*Faxonius rusticus*) populations in North America are infected with the crayfish plague disease agent (*Aphanomyces astaci*). *Freshwater Science*, **38**, 425–433. doi: 10.1086/703417

Peiró, D, Almerão, M, Delaunay, C, Jussila, J, Makkonen, J, Bouchon, D, Araujo, P and Souty-Grosset, C (2016) First detection of the crayfish plague pathogen *Aphanomyces astaci* in South America: A high potential risk to native crayfish. *Hydrobiologia*, **781**, 1–10. doi: 10.1007/s10750-016-2841-4

R Core Team (2024) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Rezinciuc, S, Sandoval-Sierra, JV, Oidtmann, B and Diéguez-Uribeondo, J (2015) The biology of crayfish plague pathogen *Aphanomyces astaci*. Current answers to most frequent questions. In Kawai T, Faulkes, Z and Scholtz G (eds). *Freshwater Crayfish: A Global Overview*. Boca Raton, FL, USA: CRC Press. pp. 182–204.

Schikora, F (1916) Die Wiederbevölkerung der deutschen Gewässer mit Krebsen. Bautzen, Germany: Emil Hübner Verlag.

Stevenson M, and Sergeant E (2024) epiR: Tools for the Analysis of Epidemiological Data. R package version 2.0.76. https://CRAN.R-project.org/package=epiR

Strand, DA, Jinnerot, T, Aspán, A, Viljamaa-Dirks, S, Heinikainen, S, Rolén, E and Vrålstad, T (2023) Molecular detection of *Aphanomyces astaci* – An improved species specific qPCR assay. *Journal of Invertebrate Pathology*, **201**, 108008. doi: 10.1016/j.jip.2023.108008

Svoboda, J, Strand, DA, Vrålstad, T, Grandjean, F, Edsman, L, Kozák, P, Kouba, A, Fristad, RF, Bahadir Koca, S and Petrusek, A (2014) The crayfish plague pathogen can infect freshwater-inhabiting crabs. *Freshwater Biology*, **59**, 918–929. doi: 10.1111/fwb.12315

Svoboda, J, Mrugała, A, Kozubíková-Balcarová, E and Petrusek, A (2017) Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: A review. *Journal of Fish Diseases*, **40**, 127–140. doi: 10.1111/jfd.12472

Swecker, CD, Jones, TD, Kilian, JV and Roberson, LF (2010) Key to the Crayfish of Maryland. Annapolis, MD, USA: Maryland Department of Natural Resources.

Thoma, RF (2022) A Naturalist's Guide to the Crayfish of Ohio. Columbus, OH, USA: Ohio Biological Survey.

Tilmans, M, Mrugała, A, Svoboda, J, Engelsma, MY, Petie, M, Soes, DM, Nutbeam-Tuffs, S, Oidtmann, B, Roessink, I and Petrusek, A (2014) Survey of the crayfish plague pathogen presence in the Netherlands reveals a new *Aphanomyces astaci* carrier. *Journal of Invertebrate Pathology*, **120**, 74–79. doi: 10.1016/j.jip.2014.06.002

Ungureanu, E, Mojžišová, M, Tangerman, M, Ion, MC, Pârvulescu, L and Petrusek, A (2020) The spatial distribution of *Aphanomyces astaci* genotypes across Europe:

Introducing the first data from Ukraine. *Freshwater Crayfish*, **25**, 77–87. doi: 10.5869/fc.2020.v25-1.077

Viljamaa-Dirks, S and Heinikainen, S (2019) A tentative new species *Aphanomyces fennicus* sp. nov. interferes with molecular diagnostic methods for crayfish plague. *Journal of Fish Diseases*, **42**, 413-422. doi: 10.1111/jfd.12955

Vrålstad, T, Knutsen, AK, Tengs, T and Holst-Jensen, A (2009) A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. *Veterinary Microbiology*, **137**, 146–155. doi: 10.1016/j.vetmic.2008.12.022

Vrålstad, T, Strand, DA, Grandjean, F, Kvellestad, A, Håstein, T, Knutsen, AK, Taugbøl, T and Skaar, I (2014) Molecular detection and genotyping of *Aphanomyces astaci* directly from preserved crayfish samples uncovers the Norwegian crayfish plague disease history. *Veterinary Microbiology*, **173**, 66–75. doi: 10.1016/j.vetmic.2014.07.008

Williams, BW, Loughman, ZJ and Lieb DA (2020) Taxonomy and Conservation Status of *Cambarus* (*Puncticambarus*) sp. in Pennsylvania. Final Report for State Wildlife Grant F16AF01305, submitted to the Pennsylvania Fish and Boat Commission, Harrisburg, Pennsylvania.

Table 1. List of studied localities, and summary of *A. astaci* detection and genotyping (separate per species and site). Results of *A. astaci* detection for each individual are included in Supplementary Table S1, detailed genotyping results, including variation at microsatellite markers, are provided in Table 3.

No: Locality [basin][1]	Latitude	Longitude	Sampling month	Crayfish sp.	Status[2]	No. inf / total	Prevalence (CI[3])	Agent level[4]	No. genotyped	mtDNA / qPCR[5]
1: East Branch White Clay Creek [D]	39.8499	−75.7838	Aug 2018	*F. limosus*	N	5 / 8	63% (24–91%)	A2–A3		
2: Chester Creek [D]	39.9314	−75.5506	Aug 2018	*F. limosus*	N	0 / 6	0% (0-46%)	N/A		
3: West Branch Red Clay Creek [D]	39.8306	−75.7217	Aug 2018	*F. limosus*	N	3 / 6	50% (12-88%)	A2-A3		
4: West Branch White Clay Creek [D]	39.7853	−75.8564	Aug 2018	*F. limosus*	N	7 / 7	100% (59–100%)	A2–A3	3	a/C
5: Broad Run #2 [D]	39.9447	−75.7012	Aug 2018	*C. bartonii*	N	2 / 2	100%	A2		
				F. limosus	N	2 / 2	100%	A2–A3		
6: Broad Run #1 [D]	39.9517	−75.7257	Aug 2018	*F. limosus*	N	1 / 3	33%	A3		
7: Broad Run at bridge [D]	39.7722	−75.7461	Aug 2018	*F. limosus*	N	1/ 7	14% (0.4–58%)	A2		
8: Furnace Run [S]	40.9584	−76.3845	Sep 2018	*C. bartonii*	N	0 / 4	0%	N/A		
9: French Creek, downstream [D]	40.1769	−75.7314	Sep 2017	*F. limosus*	N	0 / 1	0%	N/A		
				F. rusticus	I	4 / 4	100%	A2–A3		
				C. bartonii	N	1 / 1	100%	A2		
10: Crooked	41.9217	−77.1323	Nov 2021	*F. propinquu*	N?	6 / 6	100% (54–100%)	A2		

Creek [S]				*s*						
11: Crooked Creek [S]	41.8424	−77.2765	Nov 2021	*F. obscurus*	I?	2 / 9	22% (3–60%)	A2		
12: Delaware River [D]	41.6106	−75.0672	Aug 2021	*F. limosus*	N	6 / 13	46% (19–75%)	A2–A3		
13: Delaware River [D]	41.8670	−75.1934	Aug 2021	*F. limosus*	N	2 / 2	100%	A2–A3		
14: Leonard Creek [S]	41.4134	−76.0012	Aug 2021	*C. bartonii*	N	4 / 6	67% (22–96%)	A2–A3		
				F. propinquus	N?	3 / 4	75%	A2		
15: North Branch Calkins Creek [D]	41.6993	−75.1658	Aug 2021	*F. rusticus*	I	7 / 7	100% (59–100%)	A2–A3	1	a/C
16: North Branch Sugar Creek [S]	41.7957	−76.7753	Nov 2021	*F. obscurus*	I?	5 / 8	63% (24–91%)	A2–A3		
17: Freeman Run [S]	41.6699	−78.0885	Nov 2021	*C. bartonii*	N	0 / 1	0%	N/A		
				C. robustus	N?	0 / 2	0%	N/A		
18: Spring Creek [S]	40.9388	−77.7889	Oct 2021	*C. bartonii*	N	0 / 9	0% (0–34%)	N/A		
19: Spring Creek [S]	40.8833	−77.7863	Oct 2021	*F. rusticus*	I	7 / 14	50% (23–77%)	A2–A3		
20: Spring Creek [S]	40.9093	−77.7855	Oct 2021	*F. obscurus*	I?	1 / 7	14% (0.4–58%)	A2		
21: West Creek [S]	41.4825	−78.3791	Nov 2021	*C. robustus*	N?	0 / 1	0%	N/A		
				F. obscurus	I?	1 / 2	50%	A2		
22: Abrahams Creek [S]	41.3135	−75.8392	Aug 2021	*F. propinquus*	N?	5 / 10	50% (19–81%)	A2–A3	1	**b/C**

23: South Creek [S]	41.8313	−76.2745	Aug 2021	*C. bartonii*	N	0 / 9	0% (0–34%)	N/A		
24: Cowley Run [S]	41.5904	−78.1984	Nov 2021	*C. robustus*	N?	0 / 1	0%	N/A		
25: unnamed tributary to Pickering Creek [D]	40.0980	−75.5441	Sep 2021	*C. bartonii*	N	1 / 1	100%	A3	1	a/?
				F. virilis	I	2 / 2	100%	A2–A3		
26: Mill Creek [S]	41.8729	−76.9148	Nov 2021	*C. bartonii*	N	0 / 1	0%	N/A		
				F. obscurus	I?	0 / 1	0%	N/A		
27: Radley Run [D]	39.9159	−75.5943	Jul 2022	*C. bartonii*	N	6 / 6	100% (54–100%)	A2–A3	1	?/C
				F. limosus	N	5 / 5	100% (48–100%)	A2–A3		
28: Bennetts Run [D]	39.8875	−75.6287	Jul 2022	*C. bartonii*	N	3 / 5	60% (15–95%)	A2–A4	2	a/C
				F. limosus	N	4 / 7	57% (18–90%)	A2–A3	1	a/C
29: Green Tree Run [D]	40.0323	−75.4912	Jul 2022	*C. bartonii*	N	1 / 2	50%	A4	1	**a/B**
30: Van Sciver Lake [D]	40.1431	−74.8070	Jun 2022	*F. limosus*	N	0 / 1	0%	N/A		
31: Schuylkill River [D]	40.1109	−75.3445	Jul 2022	*F. rusticus*	I	8 / 29	28% (13–47%)	A2–A3	1	a/C
32: Arrowmink Creek [D]	40.0577	−75.3098	Jul 2022	*F. virilis*	I	0 / 30	0% (0–12%)	N/A		
33: Crowe Creek [D]	40.0755	−75.3854	Jul 2022	*C. bartonii*	N	0 / 6	0% (0–46%)	N/A		
34:	39.961	−75.775	Jul	*C.*	N	1 / 17	6% (0.2–29%)	A4	1	a/C

unnamed tributary of West Branch Brandywine Creek [D]	0	7	2022	*bartonii*						
35: Pine Creek #2 [D]	40.0823	−75.6136	Jul 2022	*F. virilis*	I	17 / 17	100% (80–100%)	A2–A3	3	a/C
36: Pine Creek #1 [D]	40.0605	−75.6424	Jul 2022	*F. immunis*	I	1 / 6	17% (0.4–64%)	A2		
37: East Branch Brandywine Creek [D]	39.9686	−75.6732	Aug 2022	*F. limosus*	N	0 / 10	0% (0–31%)	N/A		
38: unnamed tributary of West Branch Brandywine Creek [D]	39.9245	−75.7374	Jul 2022	*C. bartonii*	N	4 / 13	31% (9–61%)	A2–A3		
				F. limosus	N	1 / 5	20% (1–72%)	A2		
39: pond at Fort Mifflin [D]	39.8759	−75.2119	Jun 2022	*F. limosus*	N	1 / 1	100%	A3		
40: East Branch	39.9047	−75.9960	Aug 2022	*F. limosus*	N	3 / 5	60% (15–95%)	A2–A3		

Octoraro Creek [S]										
41: Chester Creek [D]	39.9274	−75.5799	Jul 2022	*C. bartonii*	N	0 / 8	0% (0–37%)	N/A		
42: Birch Run [D]	40.1188	−75.6666	Jul 2022	*C. bartonii*	N	1 / 17	6% (0.2–29%)	A4	1	a/C
43: Little Valley Creek [D]	40.0475	−75.5288	Aug 2022	*F. virilis*	I	10 / 24	42% (22–63%)	A2–A3		
44: Yellow Breeches Creek [S]	40.1434	−77.0911	Aug 2022	*C. bartonii*	N	1 / 1	100%	A2		
				F. rusticus	I	21 / 21	100% (84–100%)	A2–A4	1	a/C
45: unnamed tributary of Trout Creek [D]	40.0843	−75.4190	Jul 2022	*F. virilis*	I	12 / 15	80% (52–96%)	A2–A3		
46: Letort Spring Run [S]	40.2043	−77.1823	Aug 2022	*C. bartonii*	N	1 / 23	4% (0.1–22%)	A2		
47: unnamed tributary of Conodoguinet Creek [S]	40.2745	−77.0993	Aug 2022	*F. rusticus*	I	20 / 25	80% (59–93%)	A2–A4	2	a/C
				F. virilis	I	20 / 21	95% (76–100%)	A2–A3	3	a/C
48: unnamed tributary of Yellow	40.1811	−76.9491	Aug 2022	*F. rusticus*	I	23 / 25	92% (74–99%)	A2–A4	1	?/C
				C. bartonii	N	2 / 5	40% (5–85%)	A2–A3		

Breec hes Creek [S]								
49: French Creek, upstream [D]	40.138 1	−75.552 7	Aug 2022	*F. rusticus*	I	10 / 14	71% (42–92%)	A2–A3
				F. virilis	I	1 / 2	50%	A3

[1] Locality numbers correspond to Supplementary Table 1. Abbreviations of major river basins: [D]: Delaware, [S]: Susquehanna.

[2] Status of crayfish species at the given site: N: native; I: introduced; ?: status uncertain.

[3] 95% confidence interval (CI) of the pathogen prevalence was calculated for populations with at least 5 individuals analysed.

[4] Agent levels are provided for populations with at least one *A. astaci*-positive specimen (A2: 5 to 50 PFU, A3: 50 to 1000, A4: 1000 to 10,000 PFU per reaction). N/A: no specimen considered *A. astaci*-positive, i.e., no DNA isolate exceeding agent level A1 (<5 PFU per reaction).

[5] Genotyping results are provided for mitochondrial ribosomal markers (mtDNA; lower-case letters) according to Makkonen *et al.* (2018) and for qPCR-based genotyping assays (upper-case letters) after Di Domenico *et al.* (2021); question marks indicates when a given method was not successful. Unusual marker combinations are highlighted in bold.

Table 2. Summary of qPCR-based detection of *A. astaci* in studied crayfish. Species are ordered by the number of analysed individuals, in descending order. Spatial distribution of studied populations, and their infection status, are provided separately for each species in Fig. S1.

Species	**Populations**	**Individuals**	**Prevalence range**[1]	**Genotyping**[2]	
	A. astaci-positive / total [%]			**Individuals**	**populations**
Faxonius rusticus Rusty crayfish	8 / 8 [100%]	100 / 139 [72%]	28–100%	6	5
Cambarus bartonii Appalachian brook crayfish	13 / 20 [65%]	28 / 137 [20%]	0–100%	7	6
Faxonius virilis Virile crayfish	6 / 7 [86%]	62 / 111 [56%]	0–100%	6	2
Faxonius limosus Spiny-cheek crayfish	13 / 17 [76%]	41 / 89 [46%]	0–100%	4	2
Faxonius obscurus Allegheny crayfish	4 / 5 [80%]	9 / 27 [33%]	0–63%	–	–
Faxonius propinquus Northern clearwater crayfish	3 / 3 [100%]	14 / 20 [70%]	50–100%	1	1
Faxonius immunis Calico crayfish	1 / 1 [100%]	1 / 6 [17%]	0–17%	–	–
Cambarus robustus Big Water crayfish	0 / 3 [0%]	0 / 4 [0%]	0 %	–	–

[1] The proportion of *A. astaci*-positive populations and the pathogen prevalence estimates may be biased by sites with a low number of analysed specimens of the given species, as well as by limitations of the molecular detection methods.
[2] "Genotyping" columns summarise the total number of individuals and populations, from which we obtained at least some genotyping data.

Table 3. Detailed results of *A. astaci* genotyping for each analysed crayfish specimen and all three complementary methods: sequencing of large and small mitochondrial ribosomal subunits (mtDNA), qPCR-based detection of diagnostic nuclear markers, and amplification of nine microsatellite loci (SSR). Locality numbers correspond to those in Table 1, sample codes match Supplementary Table 1. Allele compositions of microsatellite multilocus genotypes are provided in Table 4.

Locality	Species	Sample	mtDNA[1]		qPCR[2]		SSR
			rnnL	rnnS	B	C	MLG
4: West Branch White Clay Creek	*F. limosus*	US-21	a	a	–	39.0	
		US-25	a	a	–	39.1	
		US-27	a	a	–	38.5	
15: North Branch Calkins Creek	*F. rusticus*	USA-36	a	a	–	38.2	rust2
22: Abrahams Creek	*F. propinquus*	USA-86	b	b	–	39.1	PA1
25: trib. to Pickering Creek	*C. bartonii*	USA-112	a	a	–	–	
27: Radley Run	*C. bartonii*	PA-4	N/A	N/A	–	38.9	
28: Bennetts Run	*C. bartonii*	PA-15	a	a	–	35.5	PA3
		PA-16	a	a	–	37.1	PA3
	F. limosus	PA-20	a	a	–	38.3	
29: Green Tree Run	*C. bartonii*	PA-24	a	a	36.6	–	PA2
31: Schuylkill River	*F. rusticus*	PA-51	a	a	–	39.5	
34: trib. of West Branch Brandywine Creek	*C. bartonii*	PA-102	a	a	–	36.1	PA3
35: Pine Creek #2	*F. virilis*	PA-117	a	N/A	–	38.5	
		PA-119	a	a	–	37.8	
		PA-122	a	N/A	–	40.0	
42: Birch Run	*C. bartonii*	PA-176	a	a	–	35.9	mix[3]
47: trib. of Conodoguinet Creek	*F. rusticus*	PA-291	a	a	–	38.3	
		PA-297	a	a	–	39.1	
	F. virilis	PA-302	a	a	–	39.1	PA3b
		PA-307	a	a	–	38.0	

		PA-315	a	a	–	40.0	
44: Yellow Breeches Creek	*F. rusticus*	PA-216	a	a	–	39.3	PA3c
48: trib. of Yellow Breeches Creek	*F. rusticus*	PA-326	N/A	N/A	–	38.0	PA3d

[1] N/A indicates consistent failure of amplification of the given marker.
[2] Numbers indicate cycle threshold for a given qPCR assay (B or C); dashes mark no amplification.
[3] A consistent amplification of three fragments at one of the loci suggests mixed infection (see Table 4).

Table 4. Characterization of microsatellite multilocus genotypes identified in Pennsylvania, and their comparison with *A. astaci* reference genotypes (based on Grandjean *et al.*, 2014; Panteleit *et al.*, 2019; Mojžišová *et al.*, 2020, 2024). Reference genotype codes refer to culture collections of the Norwegian Veterinary Institute, Oslo (VI) and Finnish Food Authority – Ruokavirasto, Kuopio (Evira).

MLG (*host species*)	**mtDNA**	**qPCR**	**SSR locus[1]**								
			Aast2	**Aast4**	**Aast6**	**Aast7**	**Aast9**	**Aast10**	**Aast12**	**Aast13**	**Aast14**
SSR-PA1 (*F. propinquus*)	b	C	162	87	–	215	164/182	132	–	194/202	248
SSR-PA2 (*C. bartonii*)	a	B	?	89	157	207	164/182	132	222	194	–
SSR-PA3 (*C. bartonii*)	a	C	142	87/89	148/154	191	164/182	132/142	222/226	202	248
SSR-PA3b (*F. virilis*)	a	C	142	87	148	191	164/182	132/142	226	202	248
SSR-PA3c (*F. rusticus*)	a	C	142	87/89	148	191	164/182	132/142	226	202	248
SSR-PA3d (*F. rusticus*)	N/A	C	142	87/89	148	191	?	142	226	202	–
SSR-rust2 (*F. rusticus*)	a	C	142	87	148	191	168	132	240	202	248
mix[2] (*C. bartonii*)	a	C	142/16	87/105	148/154/157[2]	191/20	164/182	132/142	222/226	194/202	248

			2				7				
Reference genotypes											
SSR-A1 (Evira6462/06)	a	A	160	103	157	207	180	142	226/240	194	246
SSR-A2 (VI03557)	a	A	160	103	157	207	180	142	–	194	246
SSR-B1 (VI03555)	b	B	142	87	148	215	164/182	132	226/240	202	248
SSR-C (VI03558)	a	C	154	87	148	191	164/168	132	226	202	248
SSR-D1 (VI03556)	d1[3]	D	138	131	148	203	180	142	234	194	250
SSR-E (Evira4805)	e	E	150	87/89	148/157	207	168/182	132/142	240	194/202	248
SSR-rust1	a	C	142	87	148	191	164/168	132	240	202	248
SSR-Up	a	B	142/150	87	148	205/215	164	132/138	226	202	248
SSR-Budapest	a	C	142/162	87/105	148/157	191/207	164/182	132/142	226	194/202	248

[1] Dashes indicate consistent lack of amplification of the given locus. Question marks (for MLGs PA2 and PA3d) indicate loci that could not be unambiguously scored.
[2] A consistent amplification of three fragments at the locus Aast6 suggests the presence of more than one *A. astaci* strain or more than one oomycete species.
[3] Mitochondrial haplotype has not been determined for this particular reference strain but this haplotype is most likely, considering its distribution across Spain (Martin-Torrijos *et al.*, 2019).

Figure 1. Distribution of studied localities in Pennsylvania (with state borders highlighted), showing detection of the crayfish plague pathogen in crayfish populations (confirmed *A. astaci* presence in light red, no detection in black). Background map is based on OpenStreetMap.

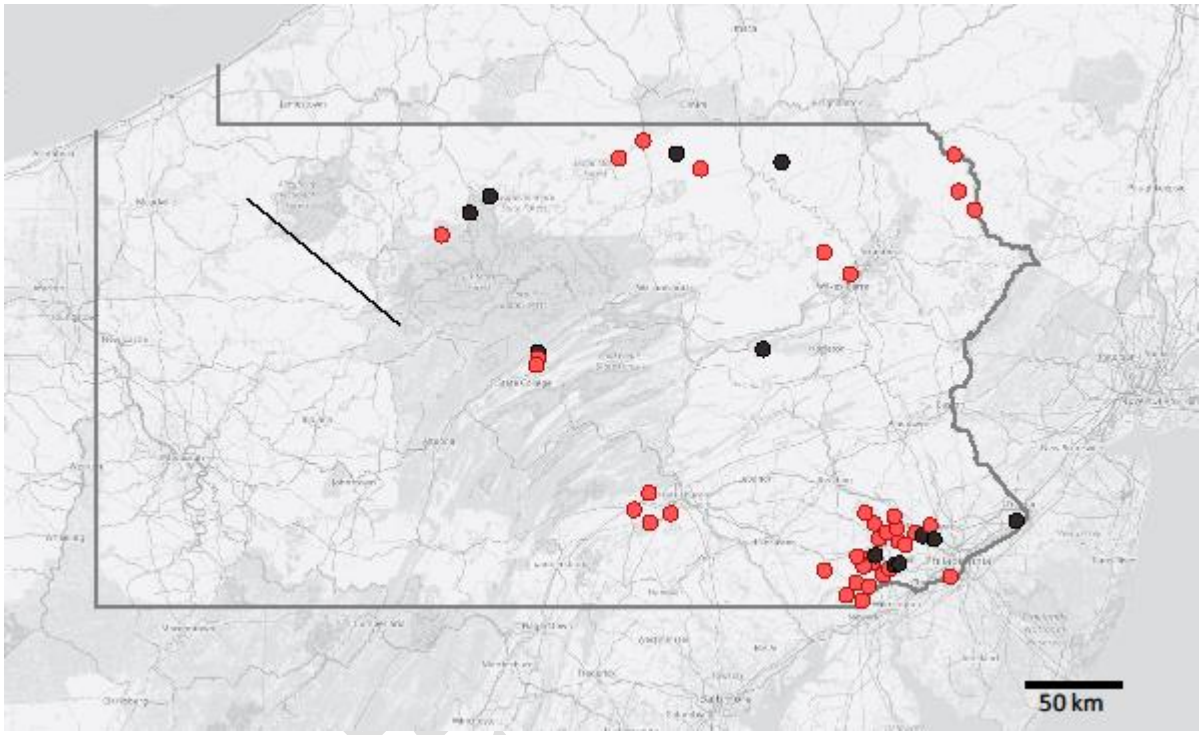

Figure 2. Results of *Aphanomyces astaci* genotyping from host crayfish with a sufficiently high infection load. Small grey dots mark localities with no detection of *A. astaci*, small black dots localities with *A. astaci* presence but no genotyping results. Larger circles indicate sites where genotyping was at least partially successful. White circles mark those where only sequencing of mitochondrial ribosomal marker(s) and/or nuclear qPCR assay were successful. Genotypes with different microsatellite multilocus genotypes (MLGs) are differentiated by colour and MLG code corresponding to Tables 3 and 4. Unless provided otherwise, a combination of mitochondrial haplotype and nuclear qPCR was a/C; the unusual mtDNA/qPCR combinations of MLGs PA1 and PA2 are marked correspondingly.