A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 109: 247–270 (2026)
DOI: 10.3897/neobiota.109.200821

Research Article

Time capsules of infection: Updating the distribution of *Aphanomyces astaci* in Croatia using archived crayfish specimens

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Abstract

The crayfish plague pathogen *Aphanomyces astaci* Schikora, 1906 is one of the most damaging invasive pathogens affecting European freshwater ecosystems, causing declines in native crayfish populations. In Croatia, crayfish plague was first reported in the late 19th century and was subsequently molecularly confirmed in 2016. However, its national distribution remains incompletely resolved because the initial molecular survey covered only a limited number of localities. To obtain an initial country-scale distribution of *A. astaci*, ethanol-preserved specimens of all seven crayfish species present in the country, collected across Croatia between 1998 and 2025, were analyzed. Tissue samples were screened for *A. astaci* using quantitative PCR (qPCR), and positive results were confirmed by Sanger sequencing. Genotype groups were assessed using pentaplex qPCR, with microsatellite genotyping and mitochondrial haplotyping applied to samples with sufficient pathogen loads. Of the 238 samples, 91 (38.2%) were *A. astaci*-positive. Pathogen presence was confirmed in both the Black Sea and Adriatic Sea drainages and across all investigated host species, extending its known distribution beyond previously surveyed localities. The integration of different genotyping methods yielded 11 interpretable consensus genotype profiles, including genotype groups A and B, SSR-UP-like, and a putatively novel genotype profile. However, additional genotypes may have remained undetected, for instance, in positive samples with low pathogen loads from *Faxonius limosus*, an invasive host widespread in Croatia and a typical carrier of genotype group E. Overall, the study shows that *A. astaci* is a widespread, long-established, and genetically diverse pathogen in Croatia, highlighting the need for coordinated, genotype-resolved national monitoring focused on invasive crayfish reservoirs and threatened indigenous populations.

Key words: Crayfish plague, invasive crayfish, molecular surveillance, native crayfish conservation, pathogen genotyping

OPEN ACCESS

Academic editor: Jamie Bojko
Received: 22 May 2026
Accepted: 14 July 2026
Published: 12 October 2026

Citation: Škugor T, Baudry T, Maguire I, Hudina S, Jemmi E, Delaunay C, Pisano SRR, Temunović M, Schmidt-Posthaus H, Bielen A (2026) Time capsules of infection: Updating the distribution of *Aphanomyces astaci* in Croatia using archived crayfish specimens. NeoBiota 109: 247–270. https://doi.org/10.3897/neobiota.109.200821

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Introduction

Freshwater crayfish are considered keystone species, ecosystem engineers, and vital constituents of freshwater food webs because of their relatively large body size, omnivorous feeding habits, and long lifespan (Usio and Townsend 2002; Reynolds and Souty-Grosset 2011; Weinländer and Füreder 2016). Once abundant

and widely distributed, indigenous crayfish species (ICS) are now facing severe population declines (Richman et al. 2015) driven by climate change, habitat and water quality deterioration, and the introduction of non-indigenous crayfish species (NICS) (Holdich et al. 2009; Richman et al. 2015).

Crayfish species have a long history of human-mediated transfer, primarily because of their use in aquaculture and the pet trade (Lodge et al. 2012). This has led to the widespread presence of NICS across Europe and worldwide (Kawai and Crandall 2016; Ion et al. 2024). Some of these species, such as *Faxonius limosus* (Rafinesque, 1817) and *Pacifastacus leniusculus* (Dana, 1852), are widespread throughout Europe and are listed as species of concern in the European Union under the Invasive Alien Species Regulation (EU 1143/2014) (European Parliament and the Council of the European Union (2014)). There are many documented instances in which NICS have outcompeted native crayfish through either competitive exclusion (Westman et al. 2002) or, more frequently, disease transmission. Specifically, they act as carriers of the crayfish plague pathogen (Richman et al. 2015), which has had a devastating impact on ICS populations (Bouallegui 2021).

The crayfish plague pathogen, *Aphanomyces astaci* Schikora, 1906, is listed among the world's 100 worst invasive species (Lowe et al. 2000) and is included in the list of notifiable aquatic animal diseases (WOAH, formerly OIE). This pathogen reproduces asexually by producing zoospores that use chemotaxis to move between hosts (Cerenius and Söderhäll 1984; Oidtmann et al. 2002; Strand et al. 2012). It is considered to originate from southeastern North America, particularly the southeastern United States, because of the uniquely high diversity of *A. astaci* haplotypes in this region (Martín-Torrijos et al. 2021). Moreover, North American crayfish species normally display low susceptibility to infection (Unestam 1969; Jussila et al. 2015; Martín-Torrijos et al. 2021), which is usually explained by long-term coevolution with the pathogen and, consequently, a rapid and continuously activated immune response to it (i.e., melanization of pathogen hyphae due to overexpression of prophenoloxidase) (Nyhlén and Unestam 1980; Cerenius et al. 2003; Jussila et al. 2015, 2021). Because this response limits but often does not eliminate the pathogen, infected NICS individuals may remain asymptomatic and act as long-term carriers of *A. astaci* (Svoboda et al. 2017).

Crayfish plague spread across Europe in two major waves: the first began in northern Italy in 1859 and had an unclear origin, whereas the second occurred in the late 1980s and was linked to introduced North American crayfish (Cornalia 1860; Alderman 1996; Jussila et al. 2015). The spread of *A. astaci* and its NICS carriers caused enormous losses of European ICS populations. For example, population declines of 50%–80% have been estimated for *Austropotamobius pallipes* (Lereboullet, 1858) since the 2000s across Europe (Collas et al. 2016). To date, five major genotype groups of *A. astaci* (A–E) have been described (Huang et al. 1994; Diéguez-Uribeondo et al. 1995; Kozubíková et al. 2011). Genotype group A is often regarded as an old European *A. astaci* lineage because it has almost always been detected in ICS (Panteleit et al. 2018), whereas the remaining genotype groups were historically described in or are commonly associated with different North American NICS (Caprioli et al. 2018; Makkonen et al. 2018; Martín-Torrijos et al. 2019; Mojžišová et al. 2020, 2024; Brady et al. 2024). Different *A. astaci* genotype groups and isolates are known to vary in phenotypic traits, such as viru-

lence, growth rate, and transmission efficiency, and in their ecological impacts on susceptible native crayfish species (Makkonen et al. 2012; Rezinciuc et al. 2014; Francesconi et al. 2024). Although crayfish plague outbreaks are well documented in ICS populations, particularly in association with generally more virulent genotype groups such as B (Vrålstad et al. 2014; Caprioli et al. 2018; Mojžišová et al. 2020), pathogen presence does not invariably result in mass mortality. European ICS may sometimes harbor chronic or subclinical infections, most often associated with genotype group A, similar to NICS that act as long-term pathogen reservoirs (Makkonen et al. 2012; Jussila et al. 2014; Svoboda et al. 2017). Moreover, some ICS populations may persist even in contact with more virulent genotype groups. As shown by infection trials, the ICS *Pontastacus leptodactylus* can be more resistant to *A. astaci* genotype group B than other native species, such as *Astacus astacus* and *Austropotamobius torrentium* (Jussila et al. 2020; unpublished data). This is consistent with field observations from the Korana River, Croatia, where *P. leptodactylus* occurs together with the NICS *P. leniusculus*. Genotype group B was detected in both species, but no crayfish plague-related mortality was documented (Maguire et al. 2016; Pavić et al. 2020; Grbin et al. 2023). However, available monitoring data indicate predominantly low *A. astaci* genotype group B loads in both species. Although infection appears to persist at a low intensity and without documented crayfish plague-related mortality, the possibility that disease outbreaks could occur in the Korana River if pathogen loads increase or environmental conditions change cannot be excluded.

In Croatia, the first crayfish plague-related mass mortalities were recorded in the late 19th century, and one further outbreak was documented in the late 1960s (Lindes 1884; Anonymous 1897, 1899, 1901; Plančić 1973). The presence of crayfish plague was first confirmed by molecular methods in 2016 (Maguire et al. 2016). The pathogen was detected in both the Black Sea and Adriatic Sea drainages and in all crayfish species present in Croatia at the time (four ICS and two NICS). Genotype groups A and B were detected: A in the ICS *Austropotamobius torrentium* (Schrank, 1803), *Astacus astacus* (Linnaeus, 1758), and *P. leptodactylus*, and B in the ICS *P. leptodactylus* and the NICS *P. leniusculus* (Maguire et al. 2016). Although this study provided important initial insight, it likely underestimated the true distribution of the pathogen in Croatia because many water bodies and regions were not covered and the molecular approaches available at the time had some limitations. In particular, *A. astaci* detection relied on the Vrålstad et al. (2009) quantitative PCR (qPCR) assay, for which specificity issues involving the closely related *A. fennicus* were later reported (Viljamaa-Dirks and Heinikainen 2019), and genotyping was based on microsatellite markers, the success of which is strongly dependent on pathogen load (Grandjean et al. 2014). Since then, more specific qPCR-based species-level detection (Strand et al. 2023), as well as qPCR-based genotyping (Di Domenico et al. 2021; Pisano et al. 2026), and complementary haplotyping approaches (Makkonen et al. 2018), have become available. Building on these advances, a historical collection of ethanol-preserved crayfish specimens collected over the past three decades was used to retrospectively assess the pathogen's distribution across the country. The aims were to (1) assess the distribution of *A. astaci* across the different biogeographical regions, drainages, and host species present in Croatia, including both ICS and NICS, and (2) identify the genotype groups present in infected crayfish populations.

Methods

Crayfish collection

Archived crayfish collected using traps or by hand from various water bodies in Croatia, including both the Black Sea (*n* = 159) and Adriatic Sea (*n* = 79) drainages, were examined. The specimens were collected over a 27-year period (1998–2025) and preserved in 96% ethanol in jars at room temperature without exposure to natural light or UV radiation (Suppl. material 2). At the time of sampling, the animals ranged from healthy-appearing individuals to dead individuals suspected to have died from crayfish plague. In total, 238 specimens were analyzed, corresponding to seven species: four ICS, *Austropotamobius torrentium* (*n* = 77), *Astacus astacus* (*n* = 58), *Austropotamobius fulcisianus* (*n* = 53), and *Pontastacus leptodactylus* (*n* = 26); and three NICS, *Faxonius limosus* (*n* = 12), *Procambarus virginalis* (*n* = 6), and *Pacifastacus leniusculus* (*n* = 6).

Tissue dissection and DNA extraction

A pool of tissue samples, i.e., the eyes, abdominal cuticle, and setae of the uropods and pereiopods (Oidtmann et al. 2006; Pisano et al. 2026), as well as melanized parts of the cuticle or exoskeleton, when present, were excised from each crayfish specimen and placed in a 2 mL Eppendorf tube together with four NucleoSpin type D beads (3 mm diameter). Tweezers and scissors were cleaned with 10% bleach followed by 96% ethanol between animals to avoid cross-contamination. Dissections and DNA extractions were also performed in a dedicated room separate from the subsequent qPCR preparation areas.

DNA extraction was performed using the Macherey-Nagel NucleoSpin DNA Insect Kit (Macherey-Nagel, Germany), following the manufacturer's instructions, with some modifications reported by Pisano et al. (2024). Briefly, all reagent volumes were tripled during the lysis step to fully submerge the tissue pool: 300 µL of Elution Buffer, 120 µL of LBP Buffer – instead of MG Buffer – and 30 µL of Proteinase K. The sample was then agitated for 7.5 min at 30 Hz using a TissueLyzer (Qiagen, Germany) before overnight incubation at 56 °C. Binding to the column was performed after adding 1,200 µL of LBP Buffer to the samples, after which the protocol followed the manufacturer's instructions. The DNA samples were stored at –20 °C until further analysis.

Aphanomyces astaci detection

The presence of *A. astaci* in the samples was investigated by qPCR using the TaqMan MGB-based protocol designed by Strand et al. (2023) to specifically detect a 131 bp fragment of the internal transcribed spacer (ITS) region of *A. astaci*. The following primers and probe were used: AphAstITS_15F, 5'-TATCCACGTGAATGTATTCTTTAT-3'; AphAstITS_145R, 5'-GCTAAGTTTATCAGTATGTTATTTA-3'; and AphAstITS_61T, 5'-6-FAM-AAGAACATCCCAGCAC-MGBNFQ-3'. The qPCR reactions were set up under a sterile hood that had previously been decontaminated using UV light treatment and were performed in a total volume of 25 µL containing 12.5 µL of TaqMan Environmental Master Mix 2.0 (Applied Biosystems, USA), 1.25 µL of each 10 µM primer (final concentration, 0.5 µM), 0.5 µL of 10 µM probe (final concentration, 0.2 µM),

1.5 µL of ddH_2O and 5 µL of DNA template. Finally, 2.5 µL of 10× Exogenous Internal Positive Control (Exo IPC) Mix Green and 0.5 µL of 50× Exo IPC DNA Yellow (Thermo Fisher, USA), both at final concentrations of 1 µM, were added to the mixture to detect possible inhibition. In addition to the tubes containing sample DNA, each qPCR run included a non-template control (NTC; PCR-grade water instead of the sample) and synthetic gBlocks Gene Fragments (Integrated DNA Technologies, USA) at a range of concentrations obtained through 10-fold serial dilution from 1×10^4 to 1×10^0 copies/µL. The gBlocks standards served as positive controls to assess qPCR efficiency and allow comparisons among runs, in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009). These synthetic double-stranded DNA fragments were 463 bp long, covered most of the ITS region of *A. astaci*, and included the primer- and probe-binding sites. Additionally, they contained a unique 3 bp sequence (ATG) to allow the detection of potential contamination and differentiation from true *A. astaci* DNA during sequencing (Suppl. material 1: fig. S1). Each qPCR plate included triplicates of 26 crayfish samples, an NTC, and five dilutions of the gBlocks standards.

The amplifications were performed using a qTOWER^3 thermocycler (Analytik Jena, Germany) with the following protocol: denaturation at 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and 58 °C for 60 s. Samples for which all three technical replicates yielded no amplification were classified as *A. astaci*-negative, whereas those showing amplification in at least one replicate were additionally examined by sequencing. Briefly, the qPCR amplicons were purified and Sanger sequenced at Microsynth, Switzerland, using the forward primer AphAstITS_15F and the company's in-house protocol. The resulting sequences, approximately 90 bp long after primer removal and trimming, were compared with the NCBI GenBank database using BLASTn to confirm species identification. A sample was considered positive for *A. astaci* only if at least one of its sequenced amplicons matched *A. astaci*; otherwise, the sample was classified as negative. The absence of gBlocks contamination was verified by examining the sequences using MEGA version 11 (Tamura et al. 2021).

Aphanomyces astaci genotyping

Three different genotyping methods were integrated: qPCR-based genotyping (Di Domenico et al. 2021; Pisano et al. 2026), mitochondrial haplotyping (Makkonen et al. 2018), and microsatellite genotyping (Grandjean et al. 2014). The final genotype assignment of the samples was obtained by combining the results of the three methods.

All DNA samples were subjected to the qPCR-based genotyping assay originally developed in two duplex and one simplex formats by Di Domenico et al. (2021) and later adapted by Pisano et al. (2026) for use in a pentaplex format. Briefly, this assay detects five different *A. astaci* genotype groups using five specific primer pairs and probes designed to amplify the anonymous locus sequences of each genotype published by Minardi et al. (2018) (GenBank accession numbers MH016383, MH016384, MH016385, MH016386, and MH016387). Initial genotyping qPCR runs using the pentaplex format occasionally yielded nonspecific amplification and irregular curve shapes that were suspected to be associated with ethanol fixation of the tissue samples. This issue was resolved by purifying all DNA samples

(20 µL) using the Qiagen DNeasy PowerClean Pro Cleanup Kit (Qiagen), following the manufacturer's instructions. The purified DNA extracts were used in subsequent genotyping reactions, which mostly yielded clear and specific amplification curves. The few remaining irregular curves were manually removed after visual inspection and were not considered true amplifications in the subsequent classification of the results. The reaction mixture contained 12.5 µL of Promega PCR Master Mix 2×, 0.625 µL of each 10 µM primer for genotype groups A–E (final concentration, 0.5 µM each), 0.25 µL of each 10 µM probe (final concentration, 0.2 µM), and 5 µL of DNA template. Each probe was labeled with a distinct fluorescent dye, as described by Di Domenico et al. (2021) and Pisano et al. (2026): FAM-BHQ-1 for A, HEX-BHQ-1 for B, ROX-BHQ-2 for C, Cy5-BHQ-2 for D, and Cy5.5-BHQ-2 for E. Reactions were performed using a CFX Opus 96 thermocycler (Bio-Rad, USA) in 96-well plates together with gBlocks fragments for each genotype group as positive controls (Pisano et al. 2026). The amplification protocol consisted of 95 °C for 2 min, followed by 50 cycles at 95 °C for 3 s and 60 °C for 30 s. Each qPCR plate included, in triplicate, 28 crayfish samples, an NTC, gBlocks standards for genotype groups A and B separately, and pooled gBlocks standards for all five genotype groups. The sequences of the gBlocks standards are provided by Pisano et al. (2026). Samples were considered positive for a specific genotype group when all three technical replicates yielded representative amplification curves.

A subset of samples with sufficient volume and pathogen load (Cq £ 30) was further genotyped by microsatellite genotyping following Grandjean et al. (2014). Briefly, PCR amplifications were performed in 10 µL reactions containing 0.5 µL of each fluorescently labeled primer (final concentration, 0.2 µM), 5 µL of Qiagen Multiplex PCR Master Mix, 1 µL of Qiagen Q-Solution, 2 µL of RNase-free water, and 1 µL of DNA. The following cycling conditions were used: 15 min at 95 °C, followed by 40 cycles at 95 °C for 30 s, 54 °C for 90 s, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. The fluorescent PCR products were then analyzed using an Applied Biosystems SeqStudio Genetic Analyzer (Waltham, USA). For samples with successful amplification of microsatellite loci, allele sizes were determined and compared with the reference profiles of Grandjean et al. (2014) to assign the samples to previously described genotype groups. Samples with multilocus profiles that did not match any available reference profile were treated as putatively novel.

Samples with sufficient volume and pathogen load (Cq £ 32) were also analyzed by mitochondrial DNA haplotyping following Makkonen et al. (2018). Briefly, each tested sample was amplified using both primer sets: mitochondrial ribosomal *rnnS* (AphSSUF, 5'-AGCACTCCGCCTGAAGAGTA-3'; and AphSSUR, 5'-GGGCGGTGTGTACAAAGTCT-3') and *rnnL* (AphLSUF, 5'-AGGCGAAAGCTTACTATGATGG-3'; and AphLSUR, 5'-CCAATTCTGTGCCACCTTCT-3'). The PCR mixture contained 12.5 µL of EmeraldAmp MAX HS PCR Master Mix (Takara Bio Inc., Japan), 1.25 µL of each 10 µM primer, 5 µL of DNA template, and 3 µL of PCR-grade water. PCR reactions were performed following the protocol for *A. astaci* haplotyping directly from crayfish cuticle samples: 95 °C for 3 min, followed by 30 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. The amplifications were examined on an agarose gel, and the PCR products were Sanger sequenced at Microsynth, Switzerland. The *rnnS* and *rnnL* sequences for each sample were edited, concatenated,

and aligned in MEGA version 11 (Tamura et al. 2021), together with 38 reference sequences (Makkonen et al. 2018; Martin-Torríjos et al. 2021). The best-fit models of evolution were determined using ModelFinder (Kalyaanamoorthy et al. 2017) based on the Bayesian information criterion (BIC). A maximum likelihood (ML) phylogenetic tree was constructed using IQ-TREE (Nguyen et al. 2015) with 1,000 standard bootstrap replicates, and nodes with bootstrap values ≥ 75 were considered supported. Samples yielding informative *rnnS* and *rnnL* sequences were assigned to haplogroups based on their positions in the phylogenetic tree relative to the reference sequences.

Data analysis

Maps were generated using QGIS v3.40.5 (QGIS Development Team 2026), with the European shapefile obtained from the Geoportal of the European Commission (EUROSTAT) and river data obtained from the HydroSHEDS program (https://www.hydrosheds.org/).

Statistical analyses and graphical presentations of the results were performed in the R environment version 4.3.2 (R Core Team 2023) using the ggplot2 package (Wickham 2016). Bar plots were generated using the geom_bar() function to visualize the numbers of *A. astaci*-positive and *A. astaci*-negative samples by crayfish species and host status (ICS vs. NICS), as well as to summarize *A. astaci* detection frequency and genotyping success over time in 5-year intervals from 1995 to 2025.

Results

Aphanomyces astaci detection

Of the 238 archived crayfish analyzed, *A. astaci* DNA was amplified and confirmed by sequencing in 91 samples (38.24% of the sample pool) (Suppl. material 2). Overall, *A. astaci* was detected across Croatia, in both the Black Sea and Adriatic Sea drainages, in both ICS and NICS, and throughout the sampling period covered by the archived material (Figs 1, 2). Information on suspected crayfish plague outbreaks was extracted from the metadata accompanying the archived samples. These records are unlikely to be exhaustive because the material was collected over many years from multiple sources, and mortality events – particularly in large rivers, where carcasses may remain undetected – are likely underreported. Nevertheless, crayfish mass mortality events suggestive of crayfish plague outbreaks were reported at four localities: Kotluša Stream in 2019 and the Krupa River in 2020, both involving *A. fulcisianus*; the Bregana River in 2025, involving *A. torrentium*; and the Otuča River in 2019, involving *A. astacus* (Fig. 2; Suppl. material 2). Compared with the first molecular detection study in Croatia by Maguire et al. (2016), the present study substantially expands the known spatial distribution of *A. astaci*, with additional positive records from previously unsurveyed or underrepresented regions and water bodies, as presented in Fig. 2. Of the 91 positive samples, 32 (35.16%) were detected in the Adriatic Sea drainage, and 59 (64.84%) were detected in the Black Sea drainage (Fig. 2).

With respect to host species, *A. astaci* was detected in all analyzed crayfish species, with relatively similar proportions of positive samples across both ICS and

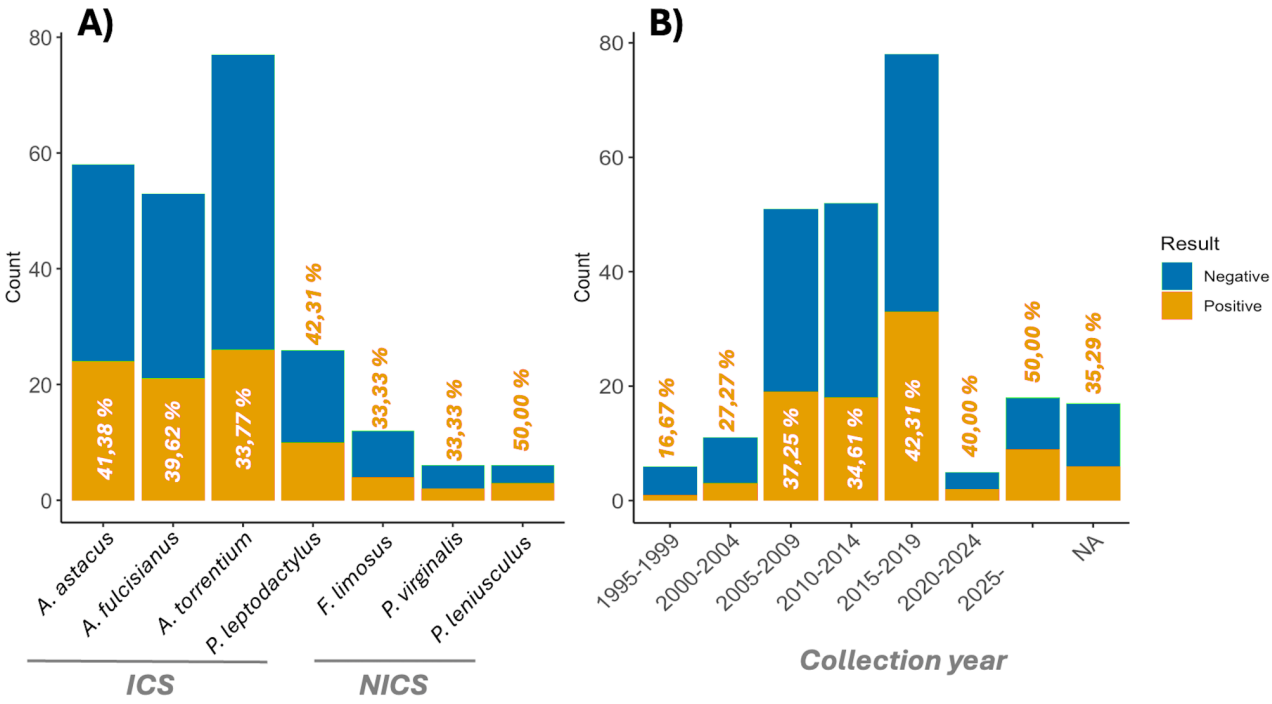

Figure 1. *Aphanomyces astaci* detection results by crayfish species (**A**) and by sampling period in 5-year intervals from 1995 to 2025 (**B**). "NA" denotes samples with no reported sampling date. The proportions of positive samples are reported above or within the bars.

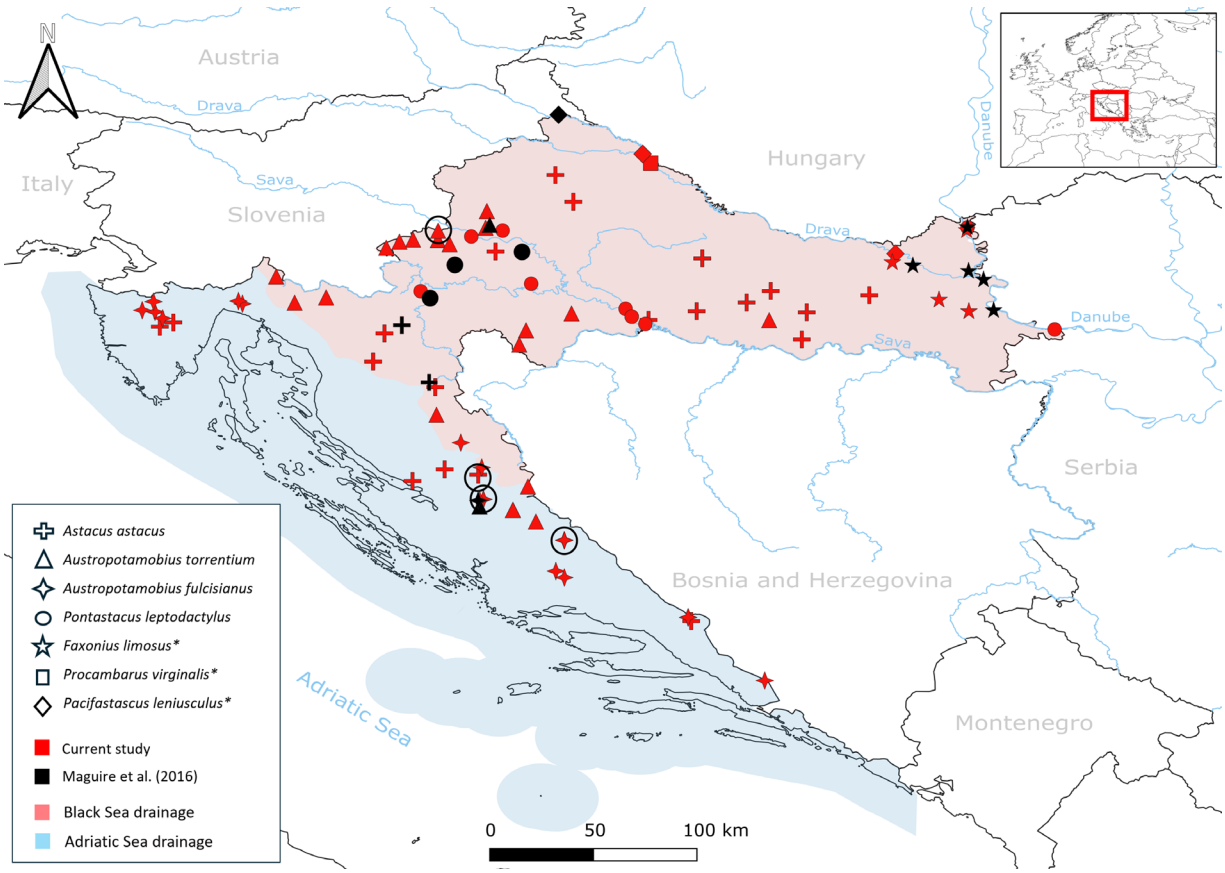

Figure 2. *Aphanomyces astaci*-positive samples detected in archived crayfish specimens collected in Croatia between 1998 and 2025 in the present study (red symbols), compared with records from the first molecular detection study of *A. astaci* in Croatia by Maguire et al. (2016) (black symbols). NICS are marked with an asterisk. Red shading indicates the Black Sea drainage, whereas blue shading indicates the Adriatic Sea drainage. Localities with suspected outbreaks are enclosed in black circles.

NICS (Fig. 1A). Among ICS, the percentage of positive samples ranged from 33.77% in *A. torrentium* to 42.31% in *P. leptodactylus*. Among NICS, the highest proportion of positive samples was observed in *P. leniusculus* (50.0%), whereas *F. limosus* and *P. virginalis* each showed 33.33% of *A. astaci*-positive samples.

Fig. 1B highlights the presence of *A. astaci* in Croatia throughout the entire sampling period, from archived specimens collected in the late 1990s to recent samples from 2025. The proportion of positive samples increased over time, ranging from 16.7% between 1995 and 1999 to more than 30% after 2005 and 50% in recently collected animals (2025). However, this apparent increase should be interpreted in consideration of the uneven sampling intensity over the years and the probable deterioration of DNA quality in ethanol-preserved specimens.

Aphanomyces astaci genotyping

Aphanomyces astaci genotyping results obtained using qPCR, haplotyping, and microsatellite marker analysis are summarized in Table 1, and most samples yielded concordant genotyping results. Overall, interpretable genotype-level information was obtained for 11 of the 91 *A. astaci*-positive samples (Table 1).

Specifically, the qPCR assay resulted in genotype group-associated amplification curves in 24 of the 238 analyzed samples (Suppl. material 2). However, only nine samples showed representative amplification curves in all three technical replicates and were therefore considered reliable qPCR-based genotype assignments: five A, three B, and one C. Lower Cq values in the *A. astaci* detection assay were more likely to yield complete 3/3 genotype assignments, whereas samples with high Cq values more frequently produced partial or no amplification during qPCR genotyping (Suppl. material 1: fig. S2). Phylogenetic analysis based on the mitochondrial *rnnS* and *rnnL* ribosomal subunits grouped the samples within mitochondrial haplogroups A (n = 8) and B (n = 2) (Table 1; Suppl. material 1: fig. S2). Microsatellite genotyping yielded complete microsatellite marker profiles for three samples – one A, one B, and one previously undocumented profile – as well as one incomplete SSR-UP-like profile (Table 1, Suppl. material 3).

Based on consensus genotype assignments, genotype group A was detected in six samples (Table 1; Fig. 3), i.e., in three ICS (*A. astacus*, *A. fulcisianus*, and

Table 1. Synthesis of positive *Aphanomyces astaci* genotyping results reported for all three methods used. Failed or unsupported genotype assignments are marked with "/"; samples that could not be analyzed because of insufficient DNA volume or pathogen load are indicated as NA. NICS are marked with an asterisk. Localities with suspected outbreaks are marked with a double asterisk.

Sample ID	Locality	Sampling year	Species	Genotyping results			Consensus genotype/profile
				qPCR	Haplotyping	Microsatellites	
356	Otuča River**	2019	*Astacus astacus*	/	A	N.A.	**A**
395	Bednja River	2018	*Astacus astacus*	A	A	N.A.	**A**
240	Kotluša Stream**	2019	*Austropotamobius fulcisianus*	A	A	A	**A**
110	Sarni Stream	2007	*Austropotamobius torrentium*	A	A	N.A.	**A**
373.1	Cerinski Stream	2019	*Austropotamobius torrentium*	A	A	N.A.	**A**
391	Babića Lake	2019	*Austropotamobius torrentium*	A	A	N.A.	**A**
315	Sloboština River	N.A.	*Astacus astacus*	B	/	/	**B**
660	Bregana River**	2025	*Austropotamobius torrentium*	B	B	B	**B**
569.2	Sava River	2007	*Pontastacus leptodactylus*	/	B	N.A.	**B-like**
664.4	Drava River	2025	*Pacifastacus leniusculus**	B	A	SSR-UP	**SSR-UP-like**
575	Danube River	2017	*Pontastacus leptodactylus*	C	A	new	**unresolved, putatively novel**

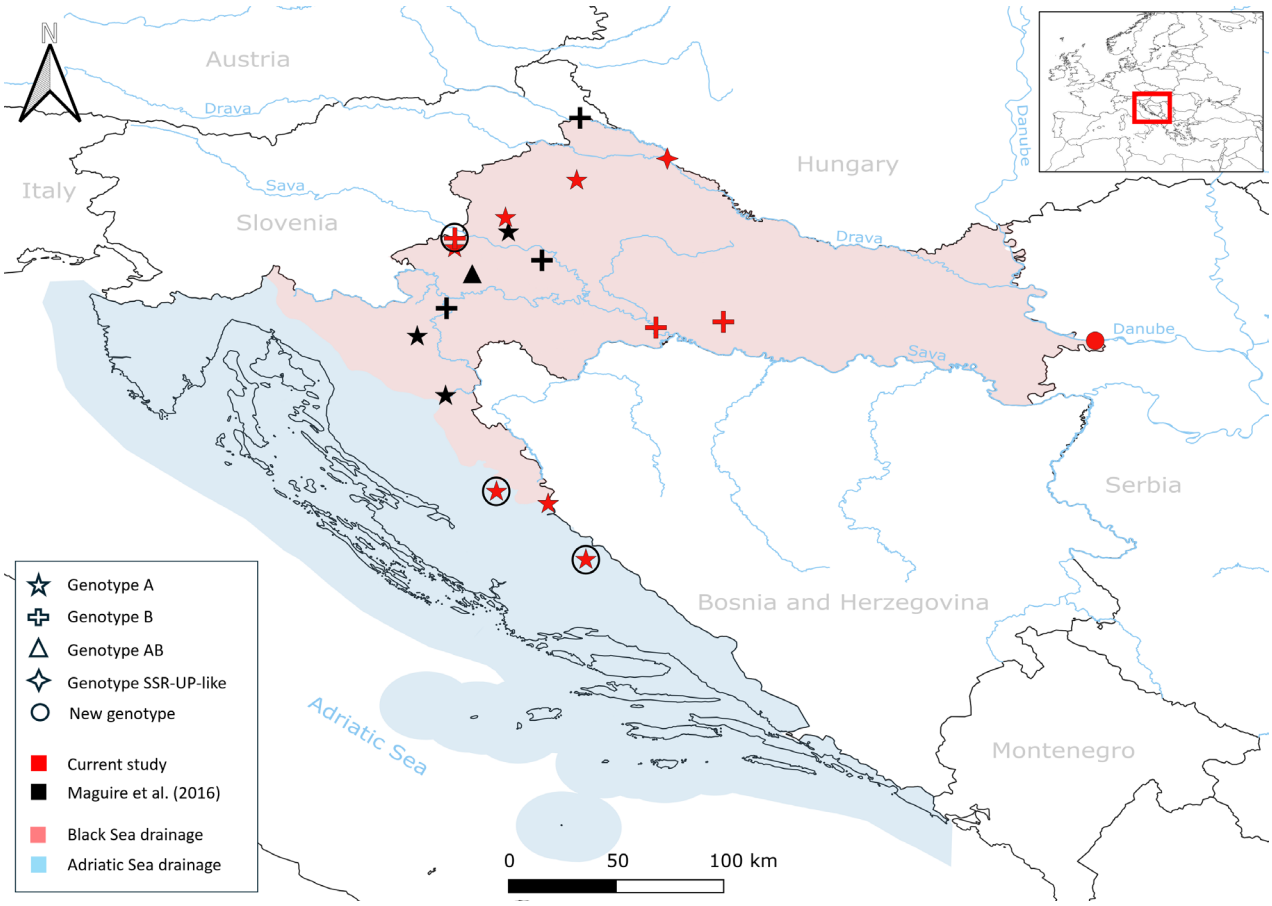

Figure 3. *Aphanomyces astaci* genotype diversity in Croatia based on the consensus genotype assignments listed in Table 1, compared with genotype records reported by Maguire et al. (2016). Red symbols indicate genotype group assignments from the present study, whereas black symbols indicate genotypes recorded by Maguire et al. (2016). The shapes of the symbols denote genotype categories. Red shading indicates the Black Sea drainage, whereas blue shading indicates the Adriatic Sea drainage. Localities with suspected outbreaks are enclosed in black circles.

A. torrentium) at several geographically separated sites spanning both the Black Sea and Adriatic Sea drainages, including two localities with suspected crayfish plague outbreaks (the Otuča River and Kotluša Stream). Genotype group B was reliably detected in two samples: *A. astacus* from the Sloboština River and *A. torrentium* from the Bregana River, with the latter reported as a mass mortality event at the time of sampling (Table 1; Fig. 3). One additional B-like assignment was obtained for *P. leptodactylus* sample 569.2 from the Sava River, based only on mitochondrial haplotyping (Table 1).

Samples 664.4 from *P. leniusculus* in the Drava River and 575 from *P. leptodactylus* in the Danube River produced discordant or inconclusive results across the genotyping methods (Table 1). Sample 664.4 yielded a genotype group B signal in the qPCR assay, whereas mitochondrial haplotyping assigned it to haplogroup A. Microsatellite genotyping produced only a partial profile, with successful amplification at three loci, although the detected alleles matched the corresponding loci of the previously described SSR-UP genotype profile (Grandjean et al. 2014). Because this marker combination – nuclear qPCR B signal, mitochondrial haplogroup A assignment, and a partial SSR-UP-compatible microsatellite profile – is consistent with the previously described SSR-UP genotype (Grandjean et al. 2014; Makkonen et al. 2018; Di Domenico et al. 2021), this sample was conservatively classified as SSR-UP-like.

Sample 575 yielded an apparent 3/3 genotype group C signal in the qPCR assay, with an additional genotype group B signal in one of the three technical replicates (Suppl. material 2). However, complementary genotyping analyses yielded nonconcordant results. Mitochondrial haplotyping placed the sample within the haplogroup A clade (Suppl. material 1: fig. S3). Microsatellite genotyping yielded a profile that did not match any of the previously described genotypes, and the obtained lengths of the microsatellite loci showed allele combinations previously documented in different reference profiles (Suppl. material 3). This sample was therefore provisionally classified as an unresolved, putatively novel genotype profile.

Discussion

Broader species-level *Aphanomyces astaci* distribution reflects sampling design, not necessarily recent expansion

This study provides the most comprehensive overview of *A. astaci* distribution in Croatia to date, obtained using ethanol-preserved crayfish specimens spanning nearly three decades and up-to-date molecular assays. Compared with Maguire et al. (2016), the dataset considerably expands the documented distribution of *A. astaci* from a limited set of surveyed populations to a broader national-scale picture, with additional records from both the Black Sea and Adriatic Sea drainages and from all ICS and NICS currently present in Croatia. However, this broader documented distribution at the species level should not be interpreted directly as evidence of recent pathogen expansion because it can largely be explained by differences in sampling strategy. The present study prioritized broad spatial coverage and analyzed one or a few archived specimens per site, which allowed a wider national-scale reconstruction of *A. astaci* distribution but limited the ability to estimate infection prevalence within individual populations. By contrast, Maguire et al. (2016) analyzed larger numbers of individuals per population, in some cases up to 16 specimens, which was better suited for detecting infection at the population level and estimating local prevalence but covered fewer sites. Consequently, some sites classified as *A. astaci*-negative in the present study may contain infected individuals that remained undetected because only a small number of specimens were analyzed per site. This also implies that the actual distribution of crayfish plague in Croatia may still be broader than documented here and remains underestimated. Overall, these two complementary datasets show that combining broad spatial screening with more intensive population-level sampling is beneficial for obtaining a comprehensive picture of pathogen distribution.

Variable distribution patterns of different *A. astaci* genotype groups

The question of whether *A. astaci* is currently expanding in Croatia is better addressed at the genotype level than through species-level detection alone, particularly because different NICS act as reservoirs of different *A. astaci* genotypes (Svoboda et al. 2017). The results suggest that genotype group A has a broad and long-term presence in Croatia, whereas evidence of possible spillover into additional ICS was found for genotype group B, which is typically associated with the signal crayfish *P. leniusculus* (Makkonen et al. 2019). Finally, an SSR-UP-like genotype and a putatively novel genotype were also detected, neither of which had been previously reported.

Historical crayfish plague outbreaks in Croatia were first reported in the late 19th and 20th centuries (Lindes 1884; Anonymous 1897, 1899, 1901; Plančić 1973), indicating that the pathogen has long been present in the country. Among *A. astaci* genotype groups, genotype group A is generally considered the earliest lineage introduced into Europe, where it has been found only in ICS. It is generally regarded as less virulent than NICS-associated genotype groups (Huang et al. 1994; Becking et al. 2015; Francesconi et al. 2024). This study provides the earliest Croatian record of genotype group A to date (sample 110, *A. torrentium* from Sarni Stream), extending its documented distribution from 2011, as reported by Maguire et al. (2016), back to 2007. Compared with Maguire et al. (2016), in which genotype group A was reported from *A. astacus* and *A. torrentium* and mainly from mountainous regions, the results expand the known host range, including the first detection in *A. fulcisianus*, and the geographical range of this genotype group in Croatia. Altogether, although its historical distribution cannot be reconstructed with certainty, the geographically broad distribution of genotype group A in multiple ICS suggests its long-term presence in Croatia. This genotype group was associated with two localities where crayfish mass mortalities suggestive of crayfish plague outbreaks were reported (*A. astacus* from the Otuča River and *A. fulcisianus* from Kotluša Stream). Although genotype group A is generally considered less virulent than genotype groups associated with NICS, previous studies have shown that it can still induce severe outbreaks and mass mortalities under favorable conditions, for instance, when host populations are exposed to environmental stressors (i.e., pollution or increased water temperature) or exhibit a compromised physiological condition (Becking et al. 2015; Francesconi et al. 2024). These observations further support the context-dependent nature of crayfish plague outcomes in European ICS populations.

In contrast to genotype group A, genotype group B is more relevant for assessing recent invasion-linked pathogen dynamics because it is typically associated with the signal crayfish *P. leniusculus* (Huang et al. 1994) and has often been linked to crayfish plague outbreaks in ICS populations (Vrålstad et al. 2014; Caprioli et al. 2018). In Croatia, *P. leniusculus* was first recorded in the Mura River in 2008 following its spread from Slovenia (Maguire et al. 2008) and in the Korana River in 2011, where it co-occurs with the native *P. leptodactylus* (Hudina et al. 2013). Maguire et al. (2016) detected genotype group B in *P. leniusculus* from the Mura River and in *P. leptodactylus* from the Korana River, i.e., within or close to the known invasion range of the signal crayfish. The results of the present study expand the documented distribution of genotype group B within the Black Sea drainage and extend its known Croatian host range to two additional ICS, *A. astacus* and *A. torrentium*, suggesting possible spillover from NICS. The detection of genotype group B in *A. torrentium* from the Bregana River in 2025 during a crayfish plague outbreak is particularly concerning. This locality lies within the Žumberak–Samoborsko gorje Nature Park, which harbors four genetically distinct evolutionarily significant units of *A. torrentium* (Lovrenčić et al. 2020). The Bregana River is outside the currently known invasion range of the signal crayfish (Dragičević et al. 2020). Similar observations, in which NICS-associated *A. astaci* genotypes were recorded in ICS populations with no previous records of NICS presence, have also been reported in other countries. For instance, NICS-associated genotype groups were reported from ICS crayfish plague outbreaks in Switzerland, the Czech Re-

public, and Ireland several years before their carrier species were first identified (Mojžišová et al. 2020; Brady et al. 2024; Pisano et al. 2026). This can be explained either by transfer via contaminated fishing gear or by spillover from previously undetected NICS upstream of the recorded outbreak, as documented previously (Pârvulescu et al. 2012). *Aphanomyces astaci*-carrying NICS might be present in low numbers but still release *A. astaci* spores that can cause disease outbreaks in highly susceptible ICS populations.

Notably, *P. leptodactylus* sample 569.2 from the Sava River was only tentatively classified as B because it was genotyped solely using mitochondrial markers, which have limited discriminatory power among the closely related A, B, and C haplotypes. In particular, the A and B haplotypes differ by only a single nucleotide in the targeted sequence, whereas A and C are identical (Makkonen et al. 2018). The cautious classification of this sample is further supported by the fact that it was collected from the Sava River in 2007, whereas the typical carrier of genotype group B, the signal crayfish *P. leniusculus*, was first recorded in Croatia in 2008 in the Mura River (Maguire et al. 2008). However, possible transfer via contaminated fishing gear cannot be excluded.

In addition to the expanded records of genotype groups A and B, two additional *A. astaci* genotype profiles were detected in Croatia for the first time: an SSR-UP-like genotype profile in *P. leniusculus* from the Drava River and an unresolved, putatively novel genotype profile in *P. leptodactylus* from the Danube River. The latter yielded a genotype group C (3/3) signal in the qPCR assay, whereas mitochondrial haplotyping placed it within haplogroup A. These results are not contradictory because the *rnnS*/*rnnL* markers do not discriminate between mitochondrial haplogroups A and C (Makkonen et al. 2018). False-positive amplification of *Aphanomyces fennicus* by the genotype group C qPCR assay is also unlikely, although such cross-reactivity has been reported previously (Di Domenico et al. 2021), because species-level sequencing of ITS amplicons confirmed *A. astaci* in this sample. However, the microsatellite profile did not match genotype group C or any other previously described reference microsatellite profile but instead contained allele combinations corresponding to different known profiles (Suppl. material 3). The combined genotyping results may therefore reflect a previously undescribed genotype profile, a mixed infection, or both. Such mixed infections with different *A. astaci* strains have been reported previously, including A/B-like microsatellite patterns in Croatian *P. leptodactylus* tissue samples (Maguire et al. 2016) and the co-occurrence of two different haplotypes in a single infected crayfish confirmed by axenic cultures after a crayfish plague outbreak in a Spanish *A. pallipes* population (Casabella-Herrero et al. 2023). Altogether, this finding should remain provisional until confirmed in additional samples or cultured isolates.

The finding of an SSR-UP-like genotype profile in the signal crayfish *P. leniusculus* from the Drava River in 2025 represents the first reported SSR-UP-like profile in a NICS host. Although the virulence of the SSR-UP genotype profile is unknown because no axenic culture is available, the finding is epidemiologically important because it has previously been associated with crayfish plague outbreaks in Czech *A. astacus* populations (Mojžišová et al. 2020) and has also been detected in chronically infected *P. leptodactylus* in the Danube River (Panteleit et al. 2018). In the present study, the Croatian sample was designated as SSR-UP-like because only a partial microsatellite profile was obtained, with three of eight loci amplified

(Suppl. material 3). Nevertheless, this result raises the possibility that signal crayfish populations may act as reservoirs or vectors of SSR-UP-like *A. astaci* strains, with potential negative effects on ICS populations with which they come into contact.

Furthermore, the genotype diversity reported here is likely still incomplete. Although *A. astaci* was detected at the species level in 91 samples, reliable or interpretable genotype-level information could be obtained for only 11 samples. This discrepancy is expected because the available genotyping approaches generally require substantially higher pathogen loads than species-level qPCR detection, as shown for microsatellite genotyping (Grandjean et al. 2014), mitochondrial haplotyping (Makkonen et al. 2018), and genotype-specific qPCR assays (Di Domenico et al. 2021; Pisano et al. 2026). This discrepancy was probably amplified by the fact that archived crayfish specimens were not primarily collected for crayfish plague diagnostics or outbreak investigations but were mostly collected during routine sampling of apparently healthy populations, in which pathogen loads were expected to be low. In addition, many samples had been preserved in ethanol for years or decades, which may reduce amplification success because of DNA degradation (Spigelman et al. 2001; Ogiso-Tanaka et al. 2025). Consistent with this, *A. astaci* detection was higher in more recently collected samples (Fig. 1B).

Therefore, the failure to detect some expected NICS-associated genotype groups should not be interpreted as evidence of their absence from Croatia. This is particularly relevant for genotype group D, which is associated with *Procambarus* spp. In Europe, the red swamp crayfish *P. clarkii* is considered the main reservoir of genotype group D, which has also been reported in the parthenogenetic marbled crayfish *P. virginalis* (Keller et al. 2014; Mrugała et al. 2015; Svoboda et al. 2017; Laffitte et al. 2024). The marbled crayfish has been present in Croatia since 2013, when it was reported from Šoderica Lake and nearby gravel pits, which remain the only known Croatian localities to date (Samardžić et al. 2014; Dobrović et al. 2021). In the present dataset, two *P. virginalis* samples from this location were *A. astaci*-positive, but their genotypes could not be resolved. A native noble crayfish (*A. astacus*) population has also been recorded in Šoderica Lake. During flooding events, the lake can become connected to the Drava River, which is part of the signal crayfish invasion area (Hudina et al. 2009; Maguire et al. 2011). This could facilitate the spread of *P. virginalis* and create opportunities for contact among native crayfish, marbled crayfish, and signal crayfish, with possible exchange of associated *A. astaci* strains. Furthermore, *Procambarus clarkii* is not yet established in Croatia, but it is widespread in several European countries (Ion et al. 2024) and has recently been reported from neighboring Slovenia in tributaries of the Sava River (Mrzelj et al. 2025). Its presence in nearby Slovenia could lead to the future establishment of *P. clarkii* in Croatia but may also pose a risk even before host establishment through the downstream transport of *A. astaci* and exposure of downstream native crayfish populations.

Similar to genotype group D, genotype group E was not detected, although its typical carrier, *F. limosus* (Kozubíková et al. 2011), has been present in Croatia since 2003 (Maguire and Gottstein-Matočec 2004). Several *F. limosus* samples in the dataset were *A. astaci*-positive but could not be genotyped because of low pathogen loads. Whether these infections represent undetected genotype group E or other *A. astaci* genotypes acquired before or after introduction requires further investigation, especially because *F. limosus* is one of the two most widespread NICS in Croatia, alongside *P. leniusculus* (Maguire et al. 2018).

Implications for national monitoring

Together, the findings highlight the need for a coordinated national monitoring framework for crayfish plague in Croatia. Such systems are well established in several European countries, including Norway, Switzerland, and Ireland (Strand et al. 2019b; Pisano et al. 2024; Sieber et al. 2024). In Switzerland, crayfish plague has been a notifiable disease since 1995, and sick, dying, or dead crayfish must be submitted for diagnosis to the reference laboratory, the Institute for Fish and Wildlife Health. In addition, targeted monitoring campaigns combining necropsy-based diagnostics, national surveys, and environmental DNA (eDNA) screening have been conducted to assess pathogen distribution and genotype diversity (Pisano et al. 2024; Pisano et al. 2026). In Norway, the national surveillance program transitioned from ethically problematic cage-based methods to eDNA monitoring, which proved more sensitive by detecting *A. astaci* weeks before crayfish mortalities and has since been officially integrated as a non-invasive, early-warning tool for monitoring crayfish plague and invasive crayfish (Strand et al. 2019a).

In Croatia, crayfish plague has been included in legislation since 1997, when the Veterinary Act listed it among infectious animal diseases whose prevention and control are of national interest (NN 70/1997 (Hrvatski Sabor 1997)). The current legal framework for the early detection, notification, and reporting of transmissible animal diseases, including crayfish plague, is provided by the Animal Health Act and the corresponding disease-notification regulation (NN 152/2022 (Hrvatski Sabor 2022); NN 55/2023 (Hrvatski Sabor 2023)). However, despite this formal basis, practical reporting and diagnostic submission of wild crayfish mortality events remain limited. This does not reflect a lack of diagnostic capacity at the official laboratory of the Croatian Veterinary Institute but rather problems with the field detection of crayfish plague outbreaks and sample transfer within the official diagnostic pathway. Crayfish mortality events may go unnoticed because field observers are not systematically trained to recognize and report suspected crayfish plague. Even when mortality is detected, diagnostic confirmation can be delayed because official sampling depends on an authorized veterinarian visiting the site and collecting suitable material, by which time carcasses may already be degraded or flushed away by water currents. Implementation should therefore be strengthened through targeted training of field personnel, clearer communication channels with authorized veterinarians, and faster submission of diagnostic material. Importantly, samples collected by protected-area staff, fish wardens, researchers, and other trained stakeholders should be better integrated into the official diagnostic pathway because relying solely on field visits by authorized veterinarians may reduce the probability of timely pathogen detection. Continuous biosafety education for fishermen, field biologists, and other water users is also needed to reduce pathogen spread through contaminated equipment or crayfish translocations.

Furthermore, monitoring priority should be given to invasion fronts, contact zones between ICS and NICS populations, and ICS populations located near or downstream of NICS reservoirs of *A. astaci* (e.g., the Drava–Mura system and the Korana–Mrežnica area for *P. leniusculus*, the Danube–Drava region for *F. limosus*, and Šoderica Lake for *P. virginalis*). Monitoring should include both *A. astaci* detection and genotype-level characterization. This is important because *A. astaci* strains differ in virulence toward ICS (Francesconi et al. 2024), and genotype information can help infer likely transmission pathways and links with specific

NICS reservoirs. As demonstrated in this study, such monitoring will require the combination of genotyping methods, especially for previously undescribed genotype profiles that might yield discordant results across different methods. Finally, tissue-based sampling should be complemented by non-invasive eDNA-based methods, in line with the One Biosecurity approach, which emphasizes integrated surveillance of invasive species and their pathogens (Hulme et al. 2023), and with ongoing EU-level efforts to standardize eDNA methods and integrate them into routine aquatic biodiversity assessments by 2030 (Hajibabaei et al. 2026). Together, these measures are needed to set up an operational *A. astaci* monitoring system in Croatia capable of timely outbreak detection, reporting, and management.

Acknowledgments

The authors are grateful to Maja Valentić (University of Zagreb Faculty of Food Technology and Biotechnology) for technical assistance during laboratory work, Matej Vucić (University of Zagreb Faculty of Science) for assistance with *A. astaci* qPCR detection, and Frédéric Grandjean (University of Poitiers) for advice regarding the microsatellite genotyping analyses. The authors also thank Pamela Nicholson of the Next Generation Sequencing Platform, University of Bern, for scientific advice during genotyping optimization.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI.
No AI tools were used in the preparation of this manuscript.

Funding

The experimental costs of this study were funded by the Croatian Science Foundation project IPCH-2024-04-6513 and the Swiss National Science Foundation project 310030L_219521. TB's salary and TŠ's mobility grant were funded by the Croatian Science Foundation through projects MOBDOL-2023-12-9061 and DOK-NPOO-2023-10-4481, respectively.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

Supplementary material 1

Supplementary figures

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Data type: docx

Explanation note: **fig. S1.** The sequence of gBlocks standard used in qPCR assay for *Aphanomyces astaci* detection (Strand et al. 2023). **fig. S2.** Influence of *Aphanomyces astaci* pathogen load, based on mean Cq-values obtained with Strand et al. (2023) quantitative PCR (qPCR) assay, on genotyping efficiency, reported as number of positive qPCR replicates using Di Domenico et al. (2021) assay (out of 3). **fig. S3.** Maximum Likelihood (ML) phylogenetic analyses of *Aphanomyces astaci* mitochondrial concatenated rnnS and rnnL sequences obtained from infected crayfish specimens (in bold) and previous studies (see Materials and Methods section).

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Link: https://doi.org/10.3897/neobiota.109.200821.suppl1

Supplementary material 2

Overview of archived crayfish specimens, including qPCR-based *Aphanomyces astaci* detection and genotyping results

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Data type: xlsx

Explanation note: Samples were classified as *A. astaci*-positive when amplification was confirmed by Sanger sequencing. Empty Cq cells indicate no amplification in the corresponding technical replicate. Genotype results are reported according to the number of positive technical replicates in the pentaplex qPCR assay of Di Domenico et al. (2021), as optimized by Pisano et al. (2026); only 3/3 results were considered reliable qPCR-based genotype assignments, whereas 1/3 and 2/3 results were treated as suspicious. "NA" indicates missing collection date or unavailable data. Localities with outbreaks are marked with double asterisk.

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Link: https://doi.org/10.3897/neobiota.109.200821.suppl2

Supplementary material 3

Microsatellite genotyping results for selected *Aphanomyces astaci*-positive samples

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Data type: xlsx

Explanation note: Allele sizes are shown for nine microsatellite loci used for direct genotyping from infected crayfish tissue. Microsatellite genotype assignments were based on comparison with reference profiles described by Grandjean et al. (2014). "N.A." indicates no amplification.

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Link: https://doi.org/10.3897/neobiota.109.200821.suppl3