NeoBiota 74: 105–127 (2022)
doi: 10.3897/neobiota.74.79087
https://neobiota.pensoft.net

RESEARCH ARTICLE

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

Long-term changes in the prevalence of the crayfish plague pathogen and its genotyping in invasive crayfish species in Czechia

Michaela Mojžišová[1], Jitka Svobodová[2], Eva Kozubíková-Balcarová[2], Eva Štruncová[2], Robin Stift[1,3], Michal Bílý[4], Antonín Kouba[5], Adam Petrusek[1]

1 *Department of Ecology, Faculty of Science, Charles University, Viničná 7, Prague 2, CZ-12844, Czech Republic* **2** *T.G. Masaryk Water Research Institute, Podbabská 30, Prague 6, CZ-16000, Czech Republic* **3** *Aquatic Ecology and Centre for Water and Environmental Research, University of Duisburg-Essen, Universitätsstraße 5, DE-45141 Essen, Germany,* **4** *Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, Suchdol, Prague, CZ-16500, Czech Republic* **5** *Faculty of Fisheries and Protection of Waters, CENAKVA, University of South Bohemia in České Budějovice, Zátiší 728/II, Vodňany, CZ-38925, Czech Republic*

Corresponding author: Adam Petrusek (petrusek@natur.cuni.cz)

Academic editor: Marcela Uliano-Silva | Received 14 December 2021 | Accepted 19 June 2022 | Published 15 July 2022

Citation: Mojžišová M, Svobodová J, Kozubíková-Balcarová E, Štruncová E, Stift R, Bílý M, Kouba A, 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. https://doi.org/10.3897/neobiota.74.79087

Abstract

The widespread presence of North American alien crayfish in Europe is a major driver of native crayfish population declines, mainly because they are chronic carriers of the oomycete *Aphanomyces astaci* responsible for crayfish plague. Screening for the crayfish plague pathogen in host populations has become a common practice across Europe, but sampling usually covers spatial but not temporal variation. Our study focuses on the current situation in Czechia, where screening for *A. astaci* was first conducted in the mid-2000s. We provide data about the distribution and prevalence of this pathogen at almost 50 sites with three host crayfish: the spiny-cheek crayfish *Faxonius limosus*, signal crayfish *Pacifastacus leniusculus*, and marbled crayfish *Procambarus virginalis.* Among these sites were 20 localities that were resampled several years (usually more than a decade) after the original screening for *A. astaci*. We did not detect any *A. astaci* infection in two studied *P. virginalis* populations but documented several new hotspots of highly infected *P. leniusculus* in Czechia, and the first site with the coexistence of the latter with *F. limosus*. Our data suggest that despite some fluctuations, *A. astaci* prevalence in North American host populations generally does not tend to change significantly over time; we only observed two cases of a significant increase and one of a significant decrease. We no longer detected *A. astaci* in several originally weakly infected popula-

tions, but our data suggest it likely still persists in these areas and threatens native crayfish populations. At the single known site in the country where *P. leniusculus* and *F. limosus* coexist, we documented the presence of the same *A. astaci* genotype group in both crayfish species, likely due to interspecific transmission of the pathogen from the former host to the latter. However, genotyping of *A. astaci* in infected host individuals still supported the link between specific pathogen genotypes and crayfish hosts, suggesting that assessment of sources of mass mortalities from the pathogen genotyping is feasible in European regions where the mutual contact of different American crayfish species is uncommon.

Keywords

Aphanomyces astaci, infection prevalence, interspecific pathogen transmission, invasive crayfish distribution, microsatellite genotyping, mitochondrial haplogroups, qPCR genotyping

Introduction

Crayfish species native to Europe face numerous threats, such as habitat loss, deteriorating water quality, overfishing or predators, with various impacts in different regions of the continent (Souty-Grosset et al. 2006). However, the key reason for declines and local extinctions of European crayfish populations is the widespread presence of invasive non-native crayfish species of North American origin (Holdich et al. 2009; Richman et al. 2015). This is partly due to their superior competitive abilities (Lindqvist and Huner 1999; van Kuijk et al. 2021), but also because these crayfish are major chronic carriers of the oomycete *Aphanomyces astaci*, a pathogen causing crayfish plague. More information on various aspects of this disease is provided in several recent reviews (Jussila et al. 2015; Rezinciuc et al. 2015; Svoboda et al. 2017; Becking et al. 2022).

Three natural host species of *A. astaci*, the spiny-cheek crayfish *Faxonius limosus*, the signal crayfish *Pacifastacus leniusculus* and the red swamp crayfish *Procambarus clarkii*, have become particularly widespread throughout Europe, but several additional alien crayfish species of the North American genera *Procambarus*, *Faxonius*, *Cambarellus* and Australasian *Cherax* have been locally introduced as well (Holdich et al. 2009; Kouba et al. 2014; Weiperth et al. 2020). *Procambarus clarkii* and *P. leniusculus* have also been introduced to other continents (*P. clarkii* being the most widespread crayfish globally; Oficialdegui et al. 2020) and along with them the crayfish plague pathogen. The introduction and spread of *A. astaci* in new regions potentially threaten local crustacean populations, including native crayfish species (Peiró et al. 2016; Mrugała et al. 2017; Martín-Torrijos et al. 2018) and those introduced for aquaculture purposes (Hsieh et al. 2016; Putra et al. 2018).

Several studies have conducted surveys on the spatial distribution and/or prevalence of chronic *A. astaci* infections in North American crayfish populations (e.g., Sandström et al. 2014; Tilmans et al. 2014; James et al. 2017b). They have shown that the prevalence of *A. astaci* may substantially differ among species and regions as well as within regions (e.g., Tilmans et al. 2014; Maguire et al. 2016; Grandjean et al. 2017). Moreover, intensive screenings in localities of coexistence with the native noble cray-

fish *Astacus astacus* have suggested that not all North American crayfish populations host this pathogen (e.g., Schrimpf et al. 2013). Although host populations reaching 100% prevalence are no exception (e.g., Kozubíková et al. 2011a; Filipová et al. 2013), they usually exhibit much lower prevalence values, and individual infection loads tend to be low as well (e.g., Maguire et al. 2016; James et al. 2017b; Panteleit et al. 2019).

In addition, there have been a few attempts, using various methodological approaches, to evaluate whether the prevalence of *A. astaci* differs over time. Nylund and Westman (2000) and Jussila et al. (2017) estimated the pathogen prevalence in *P. leniusculus* populations from gross symptoms, i.e., the presence of melanised lesions; however, these symptoms or their absence do not always correspond to results of *A. astaci* molecular detection (Kozubíková et al. 2009). Matasová et al. (2011) examined temporal changes in *A. astaci* prevalence in three *F. limosus* populations over three to six years using molecular diagnostics. They did not observe significant temporal variation in one highly infected and one very lowly infected population. However, the prevalence of an intermediately infected population decreased below the detection level over six years (Matasová et al. 2011). Unfortunately, the extent of that study was rather limited (a single host species, few populations, and a moderate time scale), thus its results cannot be generalised.

In Central and Western Europe, the key crayfish plague reservoirs are invasive North American crayfish populations (Holdich et al. 2009), although chronic *A. astaci* infections have also been documented in some native European crayfish populations (reviewed in Svoboda et al. 2017) and in the Chinese mitten crab *Eriocheir sinensis* (Schrimpf et al. 2014; Svoboda et al. 2014). However, unlike in Turkey, Finland, or Eastern European countries (Svoboda et al. 2017), no cases of chronic infections in native crayfish species in the territory of Czechia have been documented, despite dedicated efforts (Mojžišová et al. 2020). Currently, two of the three main crayfish plague carriers in Europe (*F. limosus*, *P. leniusculus*) are widespread in this country (Kouba et al. 2014; Mojžišová et al. 2020), and asymptomatic infections by *A. astaci* in Czech populations of *F. limosus*, and to a lesser extent of *P. leniusculus*, have been well documented (Kozubíková et al. 2009, 2011a; Rusch et al. 2020). The third species, *P. clarkii*, has not yet been documented from the wild in Czechia. However, populations of the marbled crayfish *Procambarus virginalis*, another proven *A. astaci* carrier (Keller et al. 2014; Mrugała et al. 2015), have recently been documented in the country, presumably originating from ornamental aquaria (Patoka et al. 2016, and unpubl. data).

Although all three invasive crayfish documented from Czechia (*F. limosus*, *P. leniusculus*, *P. virginalis*) have been included in the list of invasive alien species of the European Union concern according to Regulation (EU) No 1143/2014, their spread in the country continues, either unaided (due to active dispersal along watercourses), or due to unauthorised human-mediated introductions. As a result, new populations of all three species are being discovered (see map in Mojžišová et al. 2020).

Given that North American crayfish species pose the greatest risk as vectors of crayfish plague, country-wide screenings for the presence of *A. astaci* in their populations

have been performed in several countries. This study follows up the screening of Czech populations carried out more than a decade ago in pioneering studies that applied molecular diagnostics to study the distribution and prevalence of *A. astaci* in North American asymptomatic hosts (Kozubíková et al. 2006, 2009). In samples collected between 2004 and 2006, *F. limosus* populations showed great variability in *A. astaci* prevalence, reaching up to 100%, while *P. leniusculus* populations seemed to be infected less intensively, with prevalence not exceeding 37% (Kozubíková et al. 2009, 2011a). Since then, both species have expanded not only in the originally invaded areas, but also with numerous populations appearing in non-adjacent places, including areas bordering Austria, Germany and Poland (Štambergová et al. 2009; Mojžišová et al. 2020). Some of the newly reported borderland *P. leniusculus* populations, recently screened for *A. astaci* along with environmental DNA samples, have shown very high prevalence values (Rusch et al. 2020), in contrast to Czech populations examined previously. We presume that these highly infected populations could have been founded from sources other than the remaining Czech populations, possibly having their origin across the country border. Despite the limited distribution of *P. leniusculus* in Czechia and low *A. astaci* prevalences reported from most populations there, its importance as a local crayfish plague reservoir is also indicated by genotyping of *A. astaci* from crayfish plague outbreaks. Four mass mortalities of the native noble crayfish were caused by *A. astaci* genotypes assumed to originate from this host species (Kozubíková-Balcarová et al. 2014; M. Mojžišová, unpubl. data).

There is an assumption that distinct *A. astaci* genotype groups known from Europe are linked to their original North American crayfish carrier (for more details, see Ungureanu et al. 2020). Thus, various genotyping assays applicable on either axenic *A. astaci* cultures (Huang et al. 1994; Rezinciuc et al. 2014) or on mixed genome samples (e.g., Grandjean et al. 2014; Makkonen et al. 2018; Minardi et al. 2019; Di Domenico et al. 2021) should allow tracking the source of infection in crayfish plague outbreaks. Although a recent study using mtDNA sequencing has shown that *A. astaci* haplotypes are not host species-specific (Martín-Torrijos et al. 2021), these haplogroups likely include multiple strains that may differ from each other in variable nuclear markers targeted by other genotyping methods (e.g., RAPD: Huang et al. 1994; microsatellites: Grandjean et al. 2014) or in their physiological properties. Despite the increasing number of genotyping methods and studies applying them, there is still only limited data about *A. astaci* strains genotyped directly from North American crayfish hosts in Europe that would support the link between host species and pathogen strains co-introduced with them (reviewed in Ungureanu et al. 2020). On the contrary, some evidence for the interspecific transmission of *A. astaci* strains between North American hosts has been provided, both from captivity (Mrugała et al. 2015) and from the wild in the invaded range (James et al. 2017a).

Our study had thus three aims: (i) to update data about the *A. astaci* distribution and prevalence in Czechia including recently discovered alien crayfish populations; (ii) to investigate potential long-term temporal changes in *A. astaci* prevalence in populations of two alien crayfish species resampled after more than a decade; and (iii)

to genotype *A. astaci* in representative host individuals from multiple populations to further test the assumption that distinct *A. astaci* genotypes causing crayfish plague outbreaks in Europe are specifically linked to their North American crayfish carriers.

Materials and methods

Crayfish sampling

A total of 448 individuals of *F. limosus* from 25 sampling sites, 487 individuals of *P. leniusculus* from 23 sampling sites, and 36 individuals of *P. virginalis* from two sampling sites collected in Czechia between 2016 and 2020 (Table 1) were analysed for *A. astaci* infections. Sampling took place from various habitats, both running waters (from small streams to larger rivers) and stagnant water bodies (fishponds, reservoirs, flooded quarries, and sandpits). The sampling sites included selected localities for which past data on *A. astaci* prevalence were available from samples collected between 2004 and 2012 (most of them published in Kozubíková et al. 2011a), as well as new sites with recently reported invasive crayfish. Some of the samples collected in 2017, indicated in Table 1, have already been analysed within a study focusing on the detection of crayfish and *A. astaci* presence from environmental DNA (Rusch et al. 2020).

Crayfish specimens were collected manually or by trapping, and then preserved in 96% ethanol or deep-frozen and stored at –80 °C until further processing. We aimed to analyse 20 individuals per population, but this number sometimes could not be obtained due to low capture success, in which case we processed all available individuals. When more material from a given site was available, we occasionally analysed additional specimens to obtain more precise prevalence estimates for some populations. The number of individuals analysed per site thus ranged from five to 44 (Table 1).

Molecular detection of *A. astaci*

Crayfish tissues tested for *A. astaci* presence comprised soft abdominal cuticle and uropods; the telson was also processed from individuals with body length below 5 cm. These were homogenised by crushing after immersion in liquid nitrogen, as described in Oidtmann et al. (2006) and Kozubíková et al. (2008). DNA was extracted from up to 50 mg of the homogeneous mixture with the DNeasy tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Two negative controls consisting of 50 µl of nuclease-free water were included in each DNA extraction batch. One was kept open during manipulation with the samples to check for potential airborne laboratory contamination, another was closed to check for potential contamination of reagents. No trace of *A. astaci* DNA was detected in negative controls.

Table 1. Summary of the sampling sites and results of *A. astaci* detection in populations of alien crayfish species *F. limosus*, *P. leniusculus* and *P. virginalis* in Czechia from 2016 to 2020. Counts of individuals with agent levels above A0 (no traces of *A. astaci* DNA) are provided in parentheses. Genotyping of *A. astaci* was attempted for selected *A. astaci*-positive DNA isolates only, preferably exceeding 500 PFU. The pathogen was characterised by fragment analysis at microsatellite loci (Grandjean et al. 2014), sequencing of mitochondrial small (rnnS) and large (rnnL) ribosomal subunits (Makkonen et al. 2018) and by specific TaqMan qPCR genotyping assays (Di Domenico et al. 2021). German toponyms are provided in square brackets for transboundary watercourses. *A. astaci* prevalences in populations marked by asterisks have been previously reported in Rusch et al. (2020). Abbreviations: CI: confidence interval; SSR: multilocus genotype characterised by microsatellites; mtDNA: mitochondrial haplogroup; qPCR: genotype group determined by qPCR; NA: genotyping results from that method not available. More details on genotyping are provided in Suppl. material 1: Table S1.

Site no.	Locality	Region	River basin	Geographic coordinates	Month of sampling	Infected/ Analysed	Prevalence (95% CI)	Agent level	SSR	mtDNA	qPCR
Faxonius limosus											
1.	quarry in Starý Klíčov	Pilsen	Berounka	49.3914°N, 12.9646°E	Jun 2020	0 / 16	0% (0–21%)	–			
2.	Hracholusky reservoir*	Pilsen	Berounka	49.7976°N, 13.1024°E	Aug 2017	2 / 10	20% (3–56%)	A3	E	NA	E
3.	Lipno reservoir	South Bohemia	Vltava	48.7395°N, 14.1015°E	Aug 2017	8 / 23	35% (16–57%)	A1(4), A2(2), A3(4), A5, A6	E	e	E
4.	Barbora surface mine*	Ústí	Labe [Elbe]	50.6401°N, 13.7509°E	Aug 2017	3 / 44	7% (1– 19%)	A1(3), A2, A3(2)	NA	e	NA
5.	Zlonický brook	Central Bohemia	Vltava	50.2517°N, 13.9032°E	Jul 2017	11 / 20	55% (32–77%)	A1(2), A2(2), A3(9)	E	NA	E
6.	Vysokopecký pond	Central Bohemia	Berounka	49.6652°N, 13.9603°E	Sep 2017 Oct 2020	2 / 2 0 / 20	100% (16–100%) 0% (0–17%)	A2 –			
7.	Litavka (brook below the Vysokopecký pond)	Central Bohemia	Berounka	49.6661°N, 13.9628°E	Jul 2020	0 / 15	0% (0–22%)	–			
8.	Ohře [Eger] river	Ústí	Labe [Elbe]	50.4510°N, 14.1623°E	Sep 2017	6 / 20	30% (12–54%)	A1, A2(3), A3(3)			
9.	Vltava river (Podbaba)	Prague	Vltava	50.1183°N, 14.3931°E	Sep 2017	5 / 7	71% (29–96%)	A1, A3(5)			
10.	Vltava river (Roztoky)	Central Bohemia	Vltava	50.1454°N, 14.3974°E	Sep 2018	10 / 10	100% (69–100%)	A2(2), A3(7), A4	E	e	E
11.	Berounka river	Central Bohemia	Berounka	49.9803°N, 14.3623°E	May 2018	9 / 20	45% (23–68%)	A2(2), A3(3), A4(4)	E	e	E
12.	Vltava river under the Kořensko reservoir	South Bohemia	Vltava	49.2397°N, 14.3778°E	Aug + Sep 2019	21 / 22	95% (77–100%)	A2(2), A3(9), A4(10)	E	e	E
13.	Malše river (České Budějovice)	South Bohemia	Malše [Maltsch]	48.9752°N, 14.4709°E	Jul 2020	10 / 10	100% (69–100%)	A2, A3(7), A4(2)	NA	e	E
14.	Zlatá stoka channel*	South Bohemia	[Lainsitz]	49.0655°N, 14.6809°E	Sep 2018	1 / 8	13% (0–53%)	A1(2), A3			
15.	Baraba sandpit (Cítov)	Central Bohemia	Labe [Elbe]	50.3664°N, 14.4346°E	Aug 2019	0 / 20	0% (0–17%)	–			

Site no.	Locality	Region	River basin	Geographic coordinates	Month of sampling	Infected/ Analysed	Prevalence (95% CI)	Agent level	SSR	mtDNA	qPCR
16.	Labe [Elbe] river (Kly)*	Central Bohemia	Labe [Elbe]	50.3109°N, 14.4961°E	Jun 2017	6 / 17	35% (14–62%)	A1, A2, A3(3), A4(2)	E	e	E
17.	Kojetice quarry*	Central Bohemia	Labe [Elbe]	50.2401°N, 14.5149°E	Aug 2017	14 / 20	70% (46–88%)	A1(3), A2(14)			
18.	Konopišťský brook	Central Bohemia	Sázava	49.8401°N, 14.6795°E	Oct 2018	13 / 20	65% (41–85%)	A1(3), A2(6), A3(7)	E	NA	E
19.	Pšovka brook (Střemy)	Central Bohemia	Labe [Elbe]	50.3869°N, 14.5439°E	Jun + Jul 2020	0 / 20	0% (0–17%)	–			
20.	Pšovka brook (Harasov)*	Central Bohemia	Labe [Elbe]	50.4107°N, 14.5686°E	Aug 2017	3 / 15	20% (4–48%)	A2			
21.	Proboštská jezera sandpit	Central Bohemia	Labe [Elbe]	50.1994°N, 14.6573°E	Jul 2020	2 / 19	11% (1–33%)	A2, A3			
22.	Výmola brook (confluence with the Elbe)	Central Bohemia	Labe [Elbe]	50.1696°N, 14.7934°E	Sep 2017	0 / 20	0% (0–17%)	–			
23.	Brno reservoir	South Moravia	Dyje [Thaya]	49.2390°N, 16.5092°E	Jul 2020	8 / 20	40% (19–64%)	A1(6), A2(6), A3(2)			
24.	Prudník brook	Moravia-Silesia	Odra [Oder]	50.2982°N, 17.7437°E	Aug 2020	10 / 10	100% (69–100%)	A2, A3(7), A4(2)	E	e	E
Site with syntopic *F. limosus* (F) and *P. leniusculus* (P)											
25.	Malý Klikovský pond	South Bohemia	Lužnice [Lainsitz]	49.0971°N, 15.1433°E	Jun 2020	F: 1 / 13	8% (0–36%)	A1, A4	B	b	B
						P: 1 / 20	5% (0–25%)	A4	B	b	B
Pacifastacus leniusculus											
26.	Kouba [Chamb] brook	Pilsen	Danube [Donau]	49.3120°N, 13.0075°E	Jul 2019	0 / 20	0% (0–17%)	–			
27.	Liščí brook	Pilsen	Danube [Donau]	49.3138°N, 13.0180°E	Sep 2017	0 / 20	0% (0–17%)	–			
28.	Křesanovský brook	South Bohemia	Otava	49.0605°N, 13.7582°E	Sep 2016	0 / 22	0% (0–15%)	–			
29.	Blanice river	South Bohemia	Otava	49.1550°N, 14.1710°E	Sep 2020	0 / 20	0% (0–17%)	–			
30.	Malše [Maltsch] river (country border)*	South Bohemia	Malše [Maltsch]	48.6146°N, 14.5279°E	Aug 2017	16 / 20	80% (56–94%)	A1(3), A2(8), A3(8)	B	b	NA
31.	Pěněnský pond	South Bohemia	Lužnice [Lainsitz]	49.0988°N, 15.0412°E	May 2018	2 / 20	10% (1–32%)	A1, A2, A3	B	NA	NA
32.	Dračice brook [Kastenitzer Bach]*	South Bohemia	Lužnice [Lainsitz]	49.0056°N, 15.0951°E	Aug 2017	20 / 20	100% (83–100%)	A3(18), A4, A5	B	b	B
33.	Kačležský pond	South Bohemia	Lužnice [Lainsitz]	49.0938°N, 15.0934°E	May 2018	1 / 20	5% (0–25%)	A2			
34.	Žďárka brook*	Vysočina	Dyje [Thaya]	49.3713°N, 15.8569°E	Aug 2017	0 / 28	0% (0–12%)	–			
35.	Staviště brook*	Vysočina	Sázava	49.5672°N, 15.9448°E	Aug 2017	0 / 42	0% (0–8%)	–			
36.	Oslava river*	Vysočina	Dyje [Thaya]	49.4201°N, 15.9864°E	Apr + Aug 2017	0 / 20	0% (0–17%)	–			
37.	Prchal pond	Vysočina	Dyje [Thaya]	49.3907°N, 15.9967°E	Mar 2017	0 / 16	0% (0–21%)	–			
38.	Šípský brook	Vysočina	Dyje [Thaya]	49.3738°N, 16.0593°E	Aug 2020	0 / 20	0% (0–17%)	–			
39.	Stržek pond	Vysočina	Dyje [Thaya]	49.3782°N, 16.0840°E	Sep 2020	0 / 19	0% (0–18%)	–			

Site no.	Locality	Region	River basin	Geographic coordinates	Month of sampling	Infected/ Analysed	Prevalence (95% CI)	Agent level	SSR	mtDNA	qPCR
40.	Dolní Tis pond	Vysočina	Dyje [Thaya]	49.4366°N, 16.0985°E	Apr 2017	0 / 9	0% (0–34%)	A1			
41.	Spustík pond	Vysočina	Dyje [Thaya]	49.3829°N, 16.1308°E	Sep 2020	0 / 20	0% (0–17%)	–			
42.	brook next to Ráček I pond	Pardubice	Dyje [Thaya]	49.6688°N, 16.3339°E	Jul 2020	0 / 20	0% (0–17%)	–			
43.	Besének brook	South Moravia	Dyje [Thaya]	49.4102°N, 16.4171°E	Oct 2018	0 / 20	0% (0–17%)	–			
44.	Divoká Orlice river	Pardubice	Labe [Elbe]	50.0941°N, 16.4598°E	Jul 2020	0 / 20	0% (0–17%)	–			
45.	Bobrava river	South Moravia	Dyje [Thaya]	49.1089°N, 16.6198°E	Oct 2018	16 / 20	80% (56–94%)	A2(8), A3(7), A4	NA	b	B
				49.1090°N, 16.6116°E	Aug 2020	14 / 20	70% (46–88%)	A1(2), A2(11), A3(2), A4			
46.	Morava river	Olomouc	Morava [March]	49.3531°N, 17.3204°E	Oct 2019	0 / 14	0% (0–23%)	–			
47.	Trňák brook	Zlín	Morava [March]	49.2131°N, 17.4020°E	Oct 2018	0 / 16	0% (0–21%)	–			
Procambarus virginalis											
48.	Vršíček pond	Ústí	Labe [Elbe]	50.5536°N, 13.8264°E	Sep 2019	0 / 6	0% (0–46%)	–			
					Aug + Sep 2020	0 / 15	0% (0–22%)	–			
49.	Prostřední pond	Prague	Vltava	50.1495°N, 14.4401°E	Sep + Oct 2020	0 / 15	0% (0–22%)	–			

For detection of *A. astaci* DNA, TaqMan Minor Groove Binder (MGB) quantitative PCR (qPCR) was used on an iCycler iQ5 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The assay targeting the internal transcribed spacer 1 (ITS) in the nuclear ribosomal gene cluster was performed according to Vrålstad et al. (2009) with minor adjustments to increase specificity (as in Svoboda et al. 2014). It has been recently demonstrated that this assay cross-reacts with *Aphanomyces fennicus*, an oomycete related to *A. astaci* isolated from noble crayfish in Finland (Viljamaa-Dirks and Heinikainen 2019). Nevertheless, *A. fennicus* has not yet been reported from Central Europe, and its presence was not confirmed in any of our qPCR-positive samples that were characterised by other molecular markers allowing differentiation of *A. fennicus* and *A. astaci* (i.e., mtDNA sequencing, Makkonen et al. 2018; qPCR genotyping, Di Domenico et al. 2021). We thus interpreted positive signals in the qPCR-based screening of DNA isolates from North American host crayfish as *A. astaci* infections.

The qPCR results were evaluated using iQ5 Optical System Software version 2.0 (Bio-Rad). As the results might be biased in cases of inhibition of the PCR reaction, approx. 25% of DNA isolates were randomly selected from each population, 10-fold diluted and analysed once more for the presence of *A. astaci* DNA (Vrålstad et al. 2009; Kozubíková et al. 2011a). No sign of significant PCR inhibition was observed in any samples for which dilutions were performed.

As a positive control, we used a 251-bp long synthetically assembled DNA fragment with a sequence identical to the region of *A. astaci* internal transcribed spacer contain-

ing both primer and probe binding sites. Four standards of known concentration of the target DNA (a serial four-fold dilution with the starting concentration of 5.01×10^5 PFU) were used to quantify pathogen DNA in PCR-forming units (PFU) in a reaction according to Vrålstad et al. (2009). PFU values were used as a basis for the determination of semiquantitative levels (A0–A7), where agent levels A0 (PFU = 0) and A1 (PFU ≤ 5) were not considered *A. astaci*-positive (Vrålstad et al. 2009; Kozubíková et al. 2011a).

The *A. astaci* prevalence in analysed crayfish specimens from each locality and 95% confidence intervals were calculated in R v. 4.0.2 (R Core Team, 2020) using the function "epi.conf" from the library epiR (Stevenson et al. 2021). A potential significant change in prevalence between samples analysed before 2013 and the most recently collected ones from the same locality were compared by Fisher's exact test using the function "fisher.test". To correct for the effect of multiple testing, p-values were adjusted by Holm-Bonferroni method using the "p.adjust" function (Table 2).

Aphanomyces astaci genotyping

Three molecular assays allowing to assign *A. astaci* strains to genotype groups in mixed-genome samples – microsatellite genotyping (Grandjean et al. 2014), mtDNA sequencing (Makkonen et al. 2018), and qPCR-based genotyping (Di Domenico et al. 2021) – were performed on 20 selected chronically infected crayfish individuals from 18 sampling sites (Suppl. material 1: Table S1). These included representatives of both species from the only known Czech locality where *F. limosus* coexists in syntopy with *P. leniusculus*. In addition to recently sampled sites, we also analysed DNA isolates from individuals representing three earlier-studied *F. limosus* populations: Jickovický brook sampled in 2004 (Kozubíková et al. 2006), Pšovka brook sampled in 2005, and Prudník brook sampled in 2006 (Kozubíková et al. 2009). Another *F. limosus* individual collected from the last-mentioned site in 2020 was genotyped to check whether the presence of the genotype is consistent over time.

Infected North American crayfish tend to have relatively low *A. astaci* agent levels (e.g., James et al. 2017a), which may reduce the success of pathogen genotyping. Amplification of the target DNA fragments for available genotyping methods is usually successful for isolates with agent levels A4 and higher (over 1000 PFU as determined in the ITS-based qPCR detection of the pathogen) and for some isolates of the A3 level (Grandjean et al. 2014; Makkonen et al. 2018; Di Domenico et al. 2021). Therefore, we primarily selected DNA isolates from highly infected hosts (agent level A4 and higher) for the genotyping. We also used A3-level isolates (PFU ranging between 225 and 887) from seven localities, and attempted to increase their genotyping success by concentrating DNA by precipitation with the GlycoBlue Coprecipitant (Thermo Fisher Scientific, Invitrogen). The initial isolate volume before precipitation differed between ca 120 and 160 µl, depending on the sample availability, but each precipitated sample was diluted to a final volume of 50 µl. To save DNA isolates for genotyping, *A. astaci* DNA concentration was not quantified in those samples after the precipitation step. However, qPCR quantification of *A. astaci* ITS in other DNA isolates used in the preliminary evaluation of the suitability of this method indicated an up to four-fold increase of target DNA concentration.

Microsatellite genotyping: Variation at nine microsatellite loci was analysed to determine *A. astaci* multilocus genotypes and assign them to genotype groups as described in Grandjean et al. (2014) and amended in Mojžišová et al. (2020). Amplification using the QIAGEN Multiplex PCR Kit (Qiagen, Hilden, Germany) followed the original protocol, but was performed separately for each locus to improve genotyping success. Fragment analysis was performed on a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and allele sizes were determined in GeneMarker software version 1.95 (SoftGenetics LLC, State College, PA, USA). The results were compared with reference *A. astaci* genotypes (Grandjean et al. 2014; Mojžišová et al. 2020), in particular those originating from studies by Huang et al. (1994), Diéguez-Uribeondo et al. (1995) and Kozubíková et al. (2011b). In case of a failure to amplify some of the microsatellite loci, the given isolate was tentatively assigned to a likely genotype group if successfully scored microsatellite markers (at least three informative loci) allowed differentiating among known *A. astaci* multilocus genotypes (Suppl. material 1: Table S1). These tentative assignments were subsequently compared with the results of the remaining genotyping methods.

Sequencing of mtDNA markers: Mitochondrial small (rnnS) and large (rnnL) ribosomal subunits of *A. astaci* were amplified and sequenced according to the protocol of Makkonen et al. (2018). The amplified fragments were sequenced in both directions on a 3500 Genetic Analyzer (Applied Biosystems), chromatograms edited in Chromas 2.6 (Technelysium, Brisbane, Australia), and the obtained sequences compared with the publicly available reference sequences of known *A. astaci* haplotypes (Makkonen et al. 2018; Martín-Torrijos et al. 2018).

qPCR-based genotyping: Genotyping by qPCR targeting five anonymous nuclear markers as described in Di Domenico et al. (2021) was performed on an iCycler iQ5 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The qPCR assay followed the original protocol with only minor alterations. The assay for genotype groups B and E were run together in duplex reactions instead A/B and E/D, and the PCR cycling conditions were the same as for *A. astaci* detection described above, except for the annealing temperature, which was set to 60 °C. The results were evaluated using iQ5 Optical System Software version 2.0 (Bio-Rad).

Results

Distribution of *A. astaci* infections

A substantial difference in the proportion and spatial distribution of *A. astaci*-positive populations was observed among the tested non-native crayfish species in Czechia (Table 1, Fig. 1). Whilst no trace of the crayfish plague pathogen DNA was detected in either of the two tested *P. virginalis* populations, *A. astaci* was confirmed in 18 out of 25 sampling sites with *F. limosus* (72%), and in six out of 23 sites with *P. leniusculus* (26%).

When the crayfish plague pathogen was detected, the proportion of infected individuals among those tested ranged from 5 to 100% in populations of both host species (but note the wide confidence intervals of the prevalence estimate; Table 1). The

individual pathogen load usually reached low to moderate agent levels (A2–A4; Table 1). We did not confirm the presence of *A. astaci* in four populations of *F. limosus*, 17 populations of *P. leniusculus*, or either population of *P. virginalis* (in most cases, 20 host individuals were tested per population). In all but one case, the qPCR assay revealed no trace of *A. astaci* DNA (agent level A0) in isolates from those populations. An exception was a DNA isolate from one *P. leniusculus* individual from the Dolní Tis fishpond (site no. 40), in which a potential presence of *A. astaci* DNA in trace amounts was indicated (agent level A1, conservatively interpreted as negative according to the original recommendations by Vrålstad et al. 2009).

In cases of *F. limosus*, populations with confirmed *A. astaci* infections were scattered across the whole country (Fig. 1). Those sampled from large rivers (Elbe, Vltava, and their major tributaries) were mostly infected. Some of the populations from isolated standing water bodies (quarries, sandpits) were infected and some were not, without any apparent spatial pattern. In contrast, recent unambiguous infections of *P. leniusculus* were all restricted to the southern part of the country. In a single locality where both North American invasive species co-occurred (site no. 25), the infection was confirmed in one individual of each host, at a moderate agent level (A4). Interestingly, two of three *P. leniusculus* populations with particularly high *A. astaci* prevalence (exceeding 75%) were located in the immediate vicinity of the border with Austria (sites 30 and 32; Fig. 1).

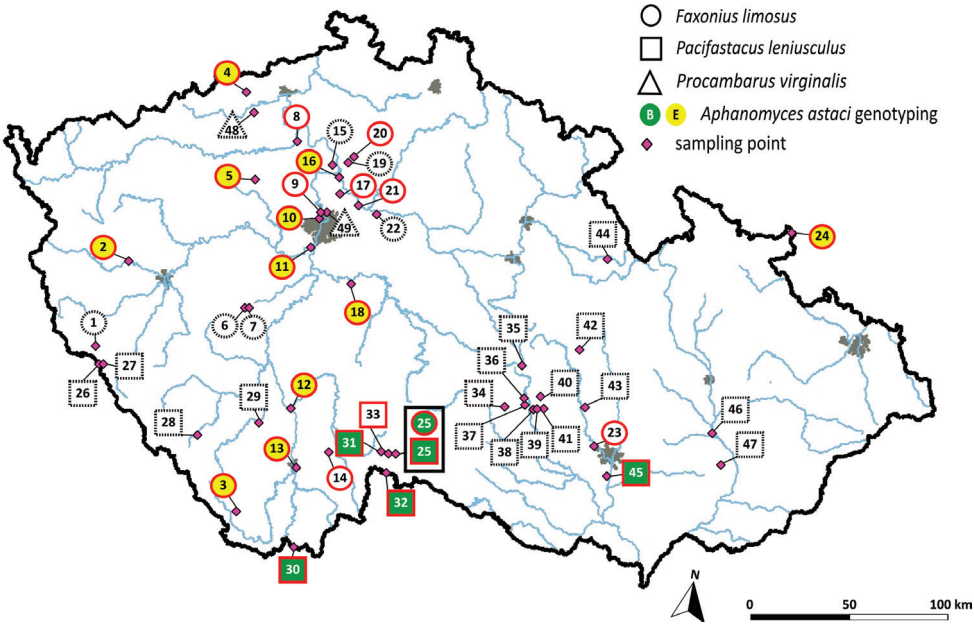

Figure 1. Distribution of populations of invasive crayfish in Czechia screened for *Aphanomyces astaci* infection between 2017 and 2020. The shape of the symbol distinguishes host species. Populations where the pathogen was detected are marked by symbols with a full red border, those without *A. astaci* detection by a black dotted border. The fill colour indicates the pathogen genotype group (dark green: group B; yellow: group E). Site no. 25 is the only locality with a known co-occurrence of *F. limosus* and *P. leniusculus*, genotype group B was detected in both host species there.

Temporal changes in *A. astaci* prevalence

A slight decrease in *A. astaci* prevalence was frequently observed over time, in a total of 13 *F. limosus* and seven *P. leniusculus* populations re-examined after several years. However, these changes were usually not significant when the number of tested individuals was considered (Table 2). A significant change in *A. astaci* prevalence was observed in only three *F. limosus* populations (Table 2). Specifically, a decrease from 61% to below the detection level in the Pšovka brook near Střemy (site no. 19), already reported by Matasová et al. (2011), was confirmed by additional sampling in 2020. In contrast, a significant increase of *A. astaci* prevalence was observed in populations from the Malše river in České Budějovice (site no. 13; from 25% in 2005 to 100% in 2020) and a flooded quarry in Kojetice (site no. 17; from 15% in 2006 to 70% in 2017).

Furthermore, contrasting results of *A. astaci* detection were obtained from the *F. limosus* population in the Vysokopecký pond (site no. 6; Table 1). Two individuals were obtained from that locality in 2017, both weakly infected by *A. astaci* (agent level A2). However, when additional crayfish were collected there three years later for analysis of a larger sample (to improve the pathogen prevalence estimate), no traces of *A. astaci* DNA were detected either in 20 individuals from that pond or in 15 individuals from the Litavka brook just below the outflow from the pond.

Aphanomyces astaci genotyping

By combining available information from the three applied genotyping methods, we successfully assigned *A. astaci* to a genotype group and/or haplogroup for all 20 tested host crayfish individuals (see details in Suppl. material 1: Table S1). The success rate of the methods nevertheless varied. All three genotyping methods were successful for all six isolates exceeding 5500 PFU in the qPCR-based *A. astaci* detection (although all nine microsatellite markers were scored for three of them only). With decreasing concentrations of target DNA in the isolates, it became increasingly common that genotyping failed for some of the methods (see Suppl. material 1: Table S1). Assignment by all three genotyping methods was possible for nine cases and by two methods in seven cases. In four cases, only one of the genotyping methods was successful. When results from multiple methods were available, they were always congruent; this was the case also when a tentative assignment to a genotype group was based on a limited number of microsatellite loci. It is noteworthy that even when an insufficient number of informative microsatellite loci were scored, the observed microsatellite allele sizes never contradicted results from other genotyping methods.

Genotyping of *A. astaci* was successful for all the isolates precipitated by GlycoBlue, in which the original agent levels in the sample were low (agent level A3). For four of these, results of two methods were available; for the remaining three, only one of the genotyping methods succeeded, without any consistent pattern (Table 1; Suppl. material 1: Table S1).

Table 2. Comparison of *A. astaci* prevalence in *F. limosus* and *P. leniusculus* populations screened before 2013 and recently. If intermediate time points are shown, only the oldest with the newest are compared statistically. Significant changes in prevalence are highlighted in bold, p-values are given after Holm-Bonferroni correction for multiple testing. Site no.: Sampling site numbers as in Table 1. CI: confidence interval; NA: data not compared statistically due to a low number of individuals in old samples.

Site no.	Locality	Month, Year	Infected/ Analysed	Prevalence (95% CI)	p-value
Faxonius limosus					
1	Lomeček quarry (Starý Klíčov)	Mar 2006†	1 / 40	2.5% (0–13%)	1
		Jun 2020	0 / 16	0% (0–21%)	
2	Hracholusky reservoir	Jun 2006†	3 / 20	15% (3–38%)	1
		Aug 2017	2 / 10	20% (3–56%)	
4	Barbora surface mine	Oct 2005†	0 / 2	0% (0–84%)	NA
		Aug 2017	3 / 44	7% (1–19%)	
7	Litavka brook	Sep 2013§	0 / 6	0% (0–46%)	NA
		Jul 2020	0 / 15	0% (0–22%)	
8	Ohře river¶	Oct 2008‡	3 / 7	43% (10–82%)	NA
		Sep 2017	6 / 20	30% (12–54%)	
12	Vltava river near Kořensko reservoir#	Apr 2004†	2 / 3	67% (9–99%)	NA
		Aug + Sep 2019	21 / 22	95% (77–100%)	
13	Malše river (České Budějovice)	Sep 2005	3 / 12	25% (6–57%)	**0.009**
		Jul 2020	10 / 10	100% (69–100%)	
15	Baraba sandpit (Cítov)	Oct 2005 + Jan 2007†	2 / 10	20% (3–56%)	1
		Aug 2019	0 / 20	0% (0–17%)	
17	Kojetice quarry	Aug 2006†	3 / 20	15% (3–38%)	**0.02**
		Aug 2017	14 / 20	70% (46–88%)	
19	Pšovka brook (Střemy)	Jun 2005†	11 / 18	61% (36–83%)	**0.0005**
		Jun + Jul 2020	0 / 20	0% (0–17%)	
20	Pšovka brook (Harasov)	2012 – 2013§	0 / 18	0% (0–19%)	1
		Aug 2017	3 / 15	20% (4–48%)	
21	Proboštská jezera sandpit	Sep 2005†	6 / 17	35% (14–62%)	1
		Oct 2019	0 / 7	0% (0–41%)	
		Jul 2020	2 / 19	10.5% (1–33%)	
24	Prudník brook	Oct 2006†	11 / 11	100% (72–100%)	1
		Aug 2020	10 / 10	100% (69–100%)	
Pacifastacus leniusculus					
26	Kouba brook	May 2006†	1 / 11	9% (0–41%)	1
		Jul 2019	0 / 20	0% (0–17%)	
29	Blanice river	Sep – Oct 2006†	2 / 8	25% (3–65%)	1
		Sep 2020	0 / 20	0% (0–17%)	
35	Staviště brook	Jul 2012‡	2 / 6	33% (4–77%)	NA
		Aug 2017	0 / 42	0% (0–8%)	
38	Šípský brook	Jun 2010‡	0 / 10	0% (0–31%)	1
		Aug 2020	0 / 20	0% (0–17%)	
39	Stržek pond	Oct 2006†	2 / 20	10% (1–32%)	1
		Sep 2020	0 / 19	0% (0–18%)	
41	Spustík pond	Oct 2006†	2 / 13	15% (2–45%)	1
		Aug 2008‡	0 / 10	0% (0–31%)	
		Sep 2020	0 / 20	0% (0–17%)	
42	Ráček pond system††	Apr + Oct 2006†	2 / 23	9% (1–28%)	1
		Jul 2020	0 / 20	0% (0–17%)	

† Results included in Kozubíková et al. (2011a). ‡ Unpublished data. § Results included in Kozubíková-Balcarová et al. (2014). ¶ Old data were obtained from a fishpond connected to the river. The distance between the sampling sites is ca 400 m. # Old data were obtained from Karlovka, tributary of the Kořensko reservoir. The difference between the sampling sites is ca 2.5 km. †† Population in an interconnected pond system. Old data were obtained from the pond Ráček II, which is about 150 m from the new sampling site (stream bypass of the pond Ráček I).

Out of 14 sampling sites with *F. limosus*, molecular markers corresponding to *A. astaci* genotype group E were detected in 13 cases. These represented localities across the whole invaded range of that species within Czechia (Fig. 1). In the Prudník brook (site no. 24), the same genotype group was confirmed both in 2006 and 2020 (Suppl. material 1: Table S1). However, at the site where *F. limosus* coexisted in syntopy with *P. leniusculus* (Malý Klikovský pond, site no. 25), genotype group B was confirmed in the infected *F. limosus* individual by all three molecular methods applied (Table 1, Fig. 1). Genotyping of *A. astaci* in infected individuals of *P. leniusculus* revealed in all cases the genotype group B; this also included an individual coexisting with *F. limosus* in the Malý Klikovský pond (Table 1, Fig. 1).

Discussion

Our data, extending the pilot study by Matasová et al. (2011), evaluated for the first time long-term changes in the prevalence of the crayfish plague pathogen in chronically infected invasive crayfish species on larger temporal and spatial scales. We statistically compared *A. astaci* prevalence in 14 sampling sites with *P. leniusculus* and *F. limosus* after more than ten years, and screened new sites with the documented presence of alien crayfish. Consistently with previous studies (Kozubíková et al. 2009, 2011a), the proportion of infected populations and prevalence values tended to be higher for *F. limosus* than for *P. leniusculus*. However, several hotspots of infected *P. leniusculus* were recently discovered in the country.

In our study, significant changes in *A. astaci* prevalence after a decade were observed only infrequently. Some fluctuations of *A. astaci* prevalence may reflect seasonality (Matasová et al. 2011), changes in host population density, or possibly the stress level to which the crayfish hosts are exposed. This might have caused the highly significant increases in *A. astaci* prevalence observed in two previously studied *F. limosus* populations. In several previously crayfish populations with low prevalences and infection levels (Kozubíková et al. 2011a), we no longer detected any trace of *A. astaci* DNA; however, the wide overlap of prevalence confidence intervals (see Table 2) indicates that the pathogen presence cannot be ruled out, and the decrease of prevalence was not significant in such cases.

A significant decrease to below the detection level in the *F. limosus* population from the Pšovka brook, already reported by Matasová et al. (2011), thus remains a notable exception. That study indicated a decrease in the prevalence of *A. astaci* to below the detection limit over six years (2004–10), and we did not detect *A. astaci* in the same brook stretch even a decade later (Table 2). Several kilometres upstream from that area, close to a zone where *F. limosus* was getting into contact with *A. astacus* as this latter species recolonised the stream, no *A. astaci* was detected in 2012 and 2013 (Kozubíková-Balcarová et al. 2014). However, we recently found *A. astaci* infections in three out of 15 tested *F. limosus* individuals there (Table 1; Rusch et al. 2020), indicating that the pathogen continues to persist within the host population and thus is an

ongoing threat to native crayfish. In fact, a sudden disappearance of *A. astacus* from a several-km-long stretch of the brook upstream of the contact zone was observed by local conservation authorities in autumn 2021 (L. Beran, pers. comm.), presumably due to an unreported crayfish plague outbreak, as predicted by Kozubíková-Balcarová et al. (2014). Therefore, any presumed disappearance of the pathogen from a previously infected host population should be considered with caution.

To obtain more reliable data about the occurrence of *A. astaci* in populations where the pathogen prevalence may be low, very high numbers of individuals per population need to be examined (see Schrimpf et al. 2013). Negatively tested individuals in the low dozens per site, as in our present study, cannot prove the absence of the pathogen. One example of a likely underestimation of *A. astaci* occurrence may be the Vysočina region (sites no. 34 to 41 in Fig. 1, Table 1), the area of the first successful introduction of *P. leniusculus* to the Czech territory (Filipová et al. 2006). We did not reliably confirm the pathogen in recently collected samples from anywhere in this region, including populations with a previously reported *A. astaci* presence (Table 2; Kozubíková et al. 2011a). Although this may possibly represent a long-term regional decrease of *A. astaci* prevalence, disappearance of the pathogen from the entire region is highly unlikely. In this context, it should be noted that in one out of nine crayfish individuals from the newly screened population in the Dolní Tis fishpond (site no. 40), a trace amount of *A. astaci* DNA was consistently detected (Table 1).

An extreme case where the absence of *A. astaci* detection likely represents a false negative result at the whole-population level might be the Vysokopecký pond (site no. 6). There, we confirmed the infection in two *F. limosus* individuals in 2017, but three years later no trace of *A. astaci* DNA was detected either in 20 individuals from the pond or in 15 individuals from the adjacent Litavka brook (Table 1). The long-term presence of the pathogen in the brook may be nevertheless assumed, as a crayfish plague outbreak caused by *A. astaci* genotype group E was confirmed in the section just below the Vysokopecký pond in 2011 (Kozubíková-Balcarová et al. 2014)

A contrasting difference between older and more recent samples, but in the opposite direction, was also observed in the Bobrava river in the south-eastern part of the country (site no. 45). Consistently high *A. astaci* prevalence (≥70%) in *P. leniusculus* was detected there in 2018 and 2020 (Table 1), but the pathogen was not detected by qPCR in 10 individuals collected approx. 12 km upstream in 2010 (E. Kozubíková-Balcarová, unpubl. data). Genotyping of the pathogen in *P. leniusculus* from this river confirmed the genotype group B, which is generally associated with this host species. Corresponding strains have been repeatedly isolated and/or genotyped from *P. leniusculus* originating in the USA (Huang et al. 1994; Makkonen et al. 2019) as well from individuals collected across its invaded range in Europe (reviewed in Ungureanu et al. 2020). Considering that the nearest locality to the Bobrava river known to host crayfish infected by *A. astaci* is inhabited by *F. limosus* (site no. 23, located within the same river basin), we presume that the long-term persistence of *A. astaci* in its original *P. leniusculus* host is a likely explanation for its recent confirmation in the Bobrava.

Genotype group B was also confirmed in all other genotyped individuals of *P. leniusculus* from Czech localities (Fig. 1). Populations of that species with sufficient infection levels to allow genotyping were located only in the southern part of the country. Except for the Bobrava river mentioned above, all those localities are close to the state border with Austria (in two cases, in the immediate vicinity). According to the species occurrence database of the Nature Conservation Agency of the Czech Republic (accessed 12/2021), they were all discovered after 2010. Nevertheless, *A. astaci* had likely been spreading from *P. leniusculus* to native crayfish earlier: four mass mortalities of native *A. astacus* caused by genotype group B have been confirmed in various regions of Czechia (Kozubíková-Balcarová et al. 2014; M. Mojžišová, unpubl.). The first was reported in 2007 from the tributary of Pěnenský pond (site no. 31), in which we confirmed the presence of infected *P. leniusculus* and genotyped the pathogen only a decade later.

In all but one case, we identified *A. astaci* genotypes that were expected to be found in European populations of their respective North American crayfish carriers (Fig. 1, Table 1). This indicates that original sources of the pathogen in crayfish mass mortalities can be reasonably assumed from genotyping results in regions where the coexistence of different North American invasive hosts is uncommon or absent. However, in a single locality in Czechia where the syntopic presence of *P. leniusculus* and *F. limosus* was discovered in 2020 (Malý Klikovský pond; site no. 25), we unambiguously identified *A. astaci* genotype group B in both host species. This indicates a likely interspecific transmission of *A. astaci* from *P. leniusculus* to *F. limosus*, as in a previously reported case from the UK where the recipient taxon was a member of the virile crayfish species complex, *Faxonius* cf. *virilis* (James et al. 2017a). Regions where multiple invasive *A. astaci* carriers coexist or may come into contact, such as the Netherlands (Tilmans et al. 2014) and Hungary (Weiperth et al. 2020), may thus yield hardly predictable host taxon – pathogen genotype combinations, making it difficult to track the origin of possible crayfish plague outbreaks.

Our experience with the inconsistent success of the applied genotyping methods confirms that characterising *A. astaci* genotypes chronically infecting their original carriers is challenging, and various methodological approaches may complement each other. In the relatively rare cases when a heavy infection of an American host is observed, all genotyping methods are likely to succeed. In already preserved material, increasing the pathogen DNA concentration in the isolate, such as with the use of the GlycoBlue Coprecipitant in our study, may increase the chance for successful genotyping. Alternatively, when live crayfish are available, the growth of the pathogen may be enhanced by their exposure to stress (as in Kozubíková et al. 2011b) or by analysis of host moults, which seems particularly promising (Martín-Torrijos et al. 2021).

Our data suggest that long-term significant changes in *A. astaci* prevalence in its North American hosts were not common within the studied populations. In several originally weakly infected populations (in particular of *P. leniusculus*) we no longer detected the pathogen, but it is likely that it persists in the area. The re-appearance of infected *F. limosus* individuals in the Pšovka brook (moreover, associated with the recent disappearance of susceptible *A. astacus* from an adjacent section of the brook) confirms that *A. astaci* prevalence at low levels (<5%) still poses a threat to local native crayfish.

The preventive rescue transfer of *A. astacus* from the Pškovka to another local watershed without alien crayfish (Kozubíková-Balcarová et al. 2014) was apparently a timely measure that contributed to the species conservation in the region. However, highly infected populations of invasive crayfish (and nearby populations of the susceptible native species) should be prioritised in any management strategies. In this context, the apparent cross-border invasion by strongly infected *P. leniusculus*, likely of different origin from the long-established weakly infected populations of that species in Czechia, is of particular concern.

Despite evidence of the apparent interspecific transmission of *A. astaci* from *P. leniusculus* to *F. limosus* at one site, our results generally support the link between specific pathogen genotypes and particular North American crayfish hosts invading European waters. This suggests that *A. astaci* genotyping is a relevant approach to tracking of sources of the pathogen in crayfish plague outbreaks in Central and Western European countries. Overall, our study highlights the importance of routine country-wide screening for relevant aquatic wildlife pathogens as an integral part of relevant conservation strategies. In the case of *A. astaci*, the screening accuracy might be improved by combining the analyses of host tissues and environmental DNA (e.g., Rusch et al. 2020; Troth et al. 2020; Sieber et al. 2022).

Acknowledgements

We thank all colleagues who helped providing the samples, in particular Jiří Hladovec and Jiří Patoka, and the Nature Conservation Agency of the Czech Republic for access to the species occurrence database. This study was funded by the Technology Agency of the Czech Republic [project no. TH02030687] and Charles University [project SVV 260436]. JS, EK-B and AP conceived the project. JS, EK-B, EŠ, AP, MB, AK and MM collected material (with additional assistance), MM, EK-B and RS performed laboratory analyses. MM and AP wrote the draft, which was then commented by all other co-authors.

References

Becking T, Kiselev A, Rossi V, Street-Jones D, Grandjean F, Gaulin E (2022) Pathogenicity of animal and plant parasitic *Aphanomyces* spp and their economic impact on aquaculture and agriculture. Fungal Biology Reviews 40: 1–18. https://doi.org/10.1016/j.fbr.2021.08.001

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

Diéguez-Uribeondo J, Huang T, Cerenius L, Söderhäll K (1995) Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii.* Mycological Research 99(5): 574–578. https://doi.org/10.1016/S0953-7562(09)80716-8

Filipová L, Petrusek A, Kozák P, Policar T (2006) *Pacifastacus leniusculus* (Dana, 1852). In: Mlíkovský J, Stýblo P (Eds) Nepůvodní druhy ve fauně a flóře České republiky [Alien species of fauna and flora of the Czech Republic]. ČSOP Praha (Prague): 239–240.

Filipová L, Petrusek A, Matasová K, Delaunay C, Grandjean F (2013) Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in population of the signal crayfish *Pacifastacus leniusculus* in France: Evaluating the threat to native crayfish. PLoS ONE 8(7): e70157. https://doi.org/10.1371/journal.pone.0070157

Grandjean F, Vrålstad T, Diéguez-Uribeondo J, Jelic M, Mangombi J, Delaunay C, Filipová L, Rezinciuc S, Kozubiková-Balcarová E, Guyonnet D, Viljamaa-Dirks S, Petrusek A (2014) Microsatellite markers for direct genotyping of the crayfish plague pathogen *Aphanomyces astaci* (Oomycetes) from infected host tissues. Veterinary Microbiology 170(3–4): 317–324. https://doi.org/10.1016/j.vetmic.2014.02.020

Grandjean F, Roques J, Delaunay C, Petrusek A, Becking T, 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(4): 541–549. https://doi.org/10.3391/ai.2017.12.4.10

Holdich DM, Reynolds JD, Souty-Grosset C, 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. https://doi.org/10.1051/kmae/2009025

Hsieh CY, Huang CW, Pan YC (2016) Crayfish plague *Aphanomyces astaci* detected in redclaw crayfish, *Cherax quadricarinatus* in Taiwan. Journal of Invertebrate Pathology 136: 117–123. https://doi.org/10.1016/j.jip.2016.03.015

Huang TS, Cerenius L, Söderhall K (1994) Analysis of genetic diversity in the crayfish plague fungus *Aphanomyces astaci*, by random amplification of polymorphic DNA. Aquaculture 126(1–2): 1–9. https://doi.org/10.1016/0044-8486(94)90243-7

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

James J, Nutbeam-Tuffs S, Cable J, Mrugała A, Viñuela-Rodriguez N, Petrusek A, Oidtmann B (2017b) The prevalence of *Aphanomyces astaci* in invasive signal crayfish from the UK and implications for native crayfish conservation. Parasitology 144(4): 411–418. https://doi.org/10.1017/S0031182016002419

Jussila J, Vrezec A, Makkonen J, Kortet R, Kokko H (2015) Invasive crayfish and their invasive diseases in Europe with the focus on the virulence evolution of the crayfish plague. In: Jussila J, Vrezec A, Makkonen J, Kortet R, Kokko H (Eds) Biological invasions in changing ecosystems: vectors, ecological impacts, management and predictions. De Gruyter (Warsaw): 183–211. https://doi.org/10.1515/9783110438666-013

Jussila J, Tiitinen V, Edsman L (2017) Chronic crayfish plague infection and eroded swimmeret syndrome in Lake Saimaa (Finland) signal crayfish. Freshwater Crayfish 23(1): 23–28. https://doi.org/10.5869/fc.2017.v23-1.23

Keller NS, Pfeiffer M, Roessink I, Schulz R, Schrimpf A (2014) First evidence of crayfish plague agent in populations of the marbled crayfish (*Procambarus fallax* forma *virginalis*). Knowledge and Management of Aquatic Ecosystems 414: 15. https://doi.org/10.1051/kmae/2014032

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

Kozubíková E, Petrusek A, Ďuriš Z, Kozák P, Geiger S, Hoffmann R, Oidtmann B (2006) The crayfish plague in the Czech Republic – Review of recent suspect cases and a pilot detection study. Bulletin Francais de la Peche et de la Pisciculture 380–381: 1313–1323. https://doi.org/10.1051/kmae:2006037

Kozubíková E, Petrusek A, Ďuriš Z, Martin MP, Diéguez-Uribeondo J, Oidtmann B (2008) The old menace is back: Recent crayfish plague outbreaks in the Czech Republic. Aquaculture 274(2–4): 208–217. https://doi.org/10.1016/j.aquaculture.2007.11.015

Kozubíková E, Filipová L, Kozák P, Ďuriš Z, Martín MP, Diéguez-Uribeondo J, Oidtmann B, Petrusek A (2009) Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in invasive American crayfishes in the Czech Republic. Conservation Biology 23(5): 1204–1213. https://doi.org/10.1111/j.1523-1739.2009.01240.x

Kozubíková E, Vrålstad T, Filipová L, Petrusek A (2011a) 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(2): 113–125. https://doi.org/10.3354/dao02411

Kozubíková E, Viljamaa-Dirks S, Heinikainen S, Petrusek A (2011b) Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague pathogen *Aphanomyces astaci*. Journal of Invertebrate Pathology 108(3): 214–216. https://doi.org/10.1016/j.jip.2011.08.002

Kozubíková-Balcarová E, Beran L, Ďuriš Z, Fischer D, Horká I, Svobodová J, Petrusek A (2014) Status and recovery of indigenous crayfish populations after recent crayfish plague outbreaks in the Czech Republic. Ethology Ecology & Evolution 26(2–3): 299–319. https://doi.org/10.1080/03949370.2014.897652

Lindqvist OV, Huner JV (1999) Life history characteristics of crayfish: what makes some of them good colonizers? In: Gherardi F, Holdich DM (Eds) Crayfish in Europe as alien species. How to make the best of a bad situation? A. A. Balkema (Rotterdam): 23–30. https://doi.org/10.1201/9781315140469-3

Maguire I, Jelić M, Klobučar G, Delaunay C, Delpy M, Grandjean F (2016) Prevalence of pathogen *Aphanomyces astaci* in freshwater crayfish populations in Croatia. Diseases of Aquatic Organisms 118(1): 45–53. https://doi.org/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, 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 26(9): 1–9. https://doi.org/10.1017/S0031182018000227

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

Martín-Torrijos L, Kawai T, Makkonen J, Jussila J, Kokko H, Diéguez-Uribeondo J (2018) Crayfish plague in Japan: A real threat to the endemic *Cambaroides japonicus.* PLoS ONE 13(4): e0195353. https://doi.org/10.1371/journal.pone.0195353

Martín-Torrijos L, Martínez-Ríos M, Casabella-Herrero G, Adams SB, Jackson CR, Diéguez-Uribeondo J (2021) Tracing the origin of the crayfish plague pathogen, *Aphanomyces astaci*, to the Southeastern United States. Scientific Reports 11(1): 9332. https://doi.org/10.1038/s41598-021-88704-8

Matasová K, Kozubíková E, Svoboda J, Jarošík V, Petrusek A (2011) Temporal variation in the prevalence of the crayfish plague pathogen (*Aphanomyces astaci*) in three Czech spiny-cheek crayfish populations. Knowledge and Management of Aquatic Ecosystems 401: 14. https://doi.org/10.1051/kmae/2011029

Minardi D, Studholme DJ, Oidtmann B, Pretto T, van der Giezen M (2019) Improved method for genotyping the causative agent of crayfish plague (*Aphanomyces astaci*) based on mitochondrial DNA. Parasitology 146(8): 1022–1029. https://doi.org/10.1017/S0031182019000283

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

Mrugała A, Kozubíková-Balcarová E, Chucholl C, Resino SC, Viljamaa-Dirks S, Vukić J, Petrusek A (2015) Trade of ornamental crayfish in Europe as a possible introduction pathway for important crustacean diseases: Crayfish plague and white spot syndrome. Biological Invasions 17(5): 1313–1326. https://doi.org/10.1007/s10530-014-0795-x

Mrugała A, Kawai T, Kozubíková-Balcarová E, Petrusek A (2017) *Aphanomyces astaci* presence in Japan: A threat to the endemic and endangered crayfish species *Cambaroides japonicus*? Aquatic Conservation 27(1): 103–114. https://doi.org/10.1002/aqc.2674

Nylund V, Westman K (2000) The prevalence of crayfish plague (*Aphanomyces astaci*) in two signal crayfish (*Pacifastacus leniusculus*) populations in Finland. Journal of Crustacean Biology 20(4): 777–785. https://doi.org/10.1163/20021975-99990099

Oficialdegui FJ, Sánchez MI, Clavero M (2020) One century away from home: How the red swamp crayfish took over the world. Reviews in Fish Biology and Fisheries 30(1): 121–135. https://doi.org/10.1007/s11160-020-09594-z

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

Panteleit J, Horvarth T, Jussila J, Makkonen J, Perry W, Schultz R, Theissinger K, 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(2): 425–433. https://doi.org/10.1086/703417

Patoka J, Buřič M, Kolář V, Bláha M, Petrtýl M, Franta P, Tropek R, Kalous L, Petrusek A, Kouba A (2016) Predictions of marbled crayfish establishment in conurbations fulfilled: Evidences from the Czech Republic. Biologia 71(12): 1380–1385. https://doi.org/10.1515/biolog-2016-0164

Peiró DF, Almerão MP, Delaunay C, Jussila J, Makkonen J, Bouchon D, Araujo PB, 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): 181–190. https://doi.org/10.1007/s10750-016-2841-4

Putra MD, Bláha M, Wardiatno Y, Krisanti M, Yonvitner, Jerikho R, Kamal MM, Mojžišová M, Bystřický PK, Kouba A, Kalous L, Petrusek A, Patoka J (2018) *Procambarus clarkii* (Girard, 1852) and crayfish plague as new threats for biodiversity in Indonesia. Aquatic Conservation 28(6): 1434–1440. https://doi.org/10.1002/aqc.2970

R Core Team (2020) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria.

Rezinciuc S, Galindo J, Montserrat J, Diéguez-Uribeondo J (2014) AFLP-PCR and RAPD-PCR evidences of the transmission of the pathogen *Aphanomyces astaci* (Oomycetes) to wild populations of European crayfish from the invasive crayfish species, *Procambarus clarkii.* Fungal Biology 118(7): 612–620. https://doi.org/10.1016/j.funbio.2013.10.007

Rezinciuc S, Sandoval-Sierra JV, Oidtmann B, Diéguez-Uribeondo J (2015) The biology of crayfish plague pathogen *Aphanomyces astaci*. Current answers to most frequent questions. In: Kawai T, Faulkes Z, Scholtz G (Eds) Freshwater Crayfish: A Global Overview. CRC Press (Boca Raton): 182–204. https://doi.org/10.1201/b18723-12

Richman NI, Bohm M, Adams SB, Alvarez F, Bergey EA, Bunn JJ, Burnham Q, Cordeiro J, Coughran J, Crandall KA, Dawkins KL, Di Stefano RJ, Doran NE, Edsman L, Eversole AG, Fureder L, Furse JM, Gherardi F, Hamr P, Holdich DM, Horwitz P, Johnston K, Jones CM, Jones JP, Jones RL, Jones TG, Kawai T, Lawler S, López-Mejía M, Miller RM, Pedraza Lara C, Reynolds JD, Richardson AM, Schultz MB, Schuster GA, Sibley PJ, Souty-Grosset C, Taylor CA, Thoma RF, Walls J, Walsh TS, Collen B (2015) Multiple drivers of decline in the global status of freshwater crayfish (Decapoda: Astacidea). Philosophical Transactions of the Royal Society B 370(1662): 20140060. https://doi.org/10.1098/rstb.2014.0060

Rusch JC, Mojžišová M, Strand DA, Svobodová J, Vrålstad T, Petrusek A (2020) Simultaneous detection of native and invasive crayfish and *Aphanomyces astaci* from environmental DNA samples in a wide range of habitats in Central Europe. NeoBiota 58: 1–32. https://doi.org/10.3897/neobiota.58.49358

Sandström A, Andersson M, Asp A, Bohman P, Edsman L, Engdahl F, Nyström P, Stenberg M, Hertonsson P, Vrålstad T, Granèli W (2014) Population collapses in introduced non-indigenous crayfish. Biological Invasions 16(9): 1961-1977. https://doi.org/10.1007/s10530-014-0641-1

Schrimpf A, Maiwald T, Vrålstad T, Schulz HK, Śmietana P, Schulz R (2013) Absence of the crayfish plague pathogen (*Aphanomyces astaci*) facilitates coexistence of European and American crayfish in central Europe. Freshwater Biology 58(6): 1116–1125. https://doi.org/10.1111/fwb.12112

Schrimpf A, Schmidt T, Schulz R (2014) Invasive Chinese mitten crab (*Eriocheir sinensis*) transmits crayfish plague pathogen (*Aphanomyces astaci*). Aquatic Invasions 9(2): 203–209. https://doi.org/10.3391/ai.2014.9.2.09

Sieber N, Hartikainen H, Krieg R, Zenker A, Vorburger C (2022) Parasite DNA detection in water samples enhances crayfish plague monitoring in asymptomatic invasive populations. Biological Invasions 24(1): 281–297. https://doi.org/10.1007/s10530-021-02644-y

Souty-Grosset C, Holdich DM, Noël P, Reynolds JD, Haffner P (2006) Atlas of crayfish in Europe. Muséum national d'Histoire naturelle (Paris): 1–188.

Štambergová M, Svobodová J, Kozubíková E (2009) Raci v České republice [Crayfish in the Czech Republic]. Agentura ochrany přírody a krajiny ČR (Prague): 1–255.

Stevenson M, Nunes T, Sanchez J, Thornton R, Reiczigel J, Robison-Cox J, Sebastiani P, Solymos P, Yoshida K, Jones G, Pirikahu S, Firestone S, Kyle R, Popp J, Jay M, Charles Reynard C (2021) epiR: An R package for the analysis of epidemiological data. R package version 2.0.19.

Svoboda J, Strand DA, Vrålstad T, Grandjean F, Edsman L, Kozák P, Kouba A, Fristad RF, Koca SB, Petrusek A (2014) The crayfish plague pathogen can infect freshwater-inhabiting crabs. Freshwater Biology 59(5): 918–929. https://doi.org/10.1111/fwb.12315

Svoboda J, Mrugała A, Kozubíková-Balcarová E, Petrusek A (2017) Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: A review. Journal of Fish Diseases 40(1): 127–140. https://doi.org/10.1111/jfd.12472

Tilmans M, Mrugała A, Svoboda J, Engelsma MY, Petie M, Soes DM, Nutbeam-Tuffs S, Oidtmann B, Roessink I, 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. https://doi.org/10.1016/j.jip.2014.06.002

Troth CR, Burian A, Mauvisseau Q, Bulling M, Nightingale J, Mauvisseau C, Sweet MJ (2020) Development and application of eDNA-based tools for the conservation of white-clawed crayfish. The Science of the Total Environment 748: 141394. https://doi.org/10.1016/j.scitotenv.2020.141394

Ungureanu E, Mojžišová M, Tangerman M, Ion MC, Pârvulescu L, Petrusek A (2020) The spatial distribution of *Aphanomyces astaci* genotypes across Europe: Introducing the first data from Ukraine. Freshwater Crayfish 25(1): 77–87. https://doi.org/10.5869/fc.2020.v25-1.077

van Kuijk T, Biesmeijer JC, van der Hoorn BB, Verdonschot PF (2021) Functional traits explain crayfish invasive success in the Netherlands. Scientific Reports 11(1): 2772. https://doi.org/10.1038/s41598-021-82302-4

Viljamaa-Dirks S, Heinikainen S (2019) A tentative new species *Aphanomyces fennicus* sp. nov. interferes with molecular diagnostic methods for crayfish plague. Journal of Fish Diseases 42(3): 413–422. https://doi.org/10.1111/jfd.12955

Vrålstad T, Knutsen AK, Tengs T, 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(1–2): 146–155. https://doi.org/10.1016/j.vetmic.2008.12.022

Weiperth A, Bláha M, Szajbert B, Seprős R, Bányai Z, Patoka J, Kouba A (2020) Hungary: A European hotspot of non-native crayfish biodiversity. Knowledge and Management of Aquatic Ecosystems 421: 43. https://doi.org/10.1051/kmae/2020035

Supplementary material 1

Table S1

Authors: Michaela Mojžišová, Jitka Svobodová, Eva Kozubíková-Balcarová, Eva Štruncová, Robin Stift, Michal Bílý, Antonín Kouba, Adam Petrusek

Data type: genotyping

Explanation note: Detailed results of *Aphanomyces astaci* genotyping in individual DNA isolates. Allele sizes for each microsatellite locus are provided for all analysed samples and for relevant pathogen reference genotypes. Strains representing genotype groups B and E and highlighted in bold.

Link: https://doi.org/10.3897/neobiota.74.79087.suppl1