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Detekcia a variabilita patogénu račieho moru vo vybraných populáciách rakov

Detection and variation of the crayfish plague pathogen in selected crayfish populations

Diplomová práce

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Obsah

Abstrakt

Račí mor je tzv. „emerging disease“, ktorej pôvodcom je oomycéta *Aphanomyces astaci*, patogén zaradený medzi 100 najhorších invazívnych nepôvodných druhov na svete. Introdukcia tohto patogénu zo Severnej Ameriky do Európy v druhej polovici 19. storočia, viedla ku kolapsom pôvodných európskych populácii rakov. V dnešnej dobre je *A. astaci* rozšírený v Európe a rozšíril sa i do iných častí sveta a ohrozuje všetky vnímavé druhy rakov neseveroamerického pôvodu. Ciele tejto diplomovej práce boli 1) poskytnúť informácie o úhynoch rakov na račí mor dokumentovaných v nedávnej dobe a pomocou mikrosatelitových a mtDNA markerov odhaliť, ktoré genotypové skupiny ich spôsobili; 2) otestovať zdravo vyzerajúce pôvodné raky pre potenciálny výskyt chronických infekcii spôsobených *A. astaci* v Česku. Medzi rokmi 2016 až 2018 bolo potvrdených šesť nových úhynov na račí mor, ktoré zahrňovali najmenej päť rôznych kmeňov *A. astaci*. Moje výsledky poskytujú prvý dôkaz o tom, že genotypová skupina D spôsobila masové úhyny *Astacus astacus* a *Austropotamobius torrentium* v Česku. Sekvenovaním mtDNA boli odhalené dva rôzne haplotypy z D haploskupiny, čo naznačuje dva nezávislé zdroje infekcie pravdepodobne, buď z okrasných rakov prítomných v krajine alebo sa rozšírili zo susediacich krajín. Genotypová skupina A bola zaznamenaná u dvoch úhynov raka *A. astacus* a genotypová skupina E u jedného úhynu raka *A. torrentium*. Po 13 rokoch od prvého výskytu v Česku bol znovu identifikovaný genotyp Up pri úhyne *A. astacus*. V 15 testovaných populáciách pôvodných rakov nebol pozorovaný žiadny prípad chronickej infekcie spôsobenej *A. astaci*. Zdá sa, že tento jav nie je v Česku príliš bežný; jeho výskyt však nemožno vylúčiť. Okrem týchto výsledkov zhrnutých v práci vo forme rukopisu, prikladám v prílohách dve publikované práce, v ktorých som prispela ako spoluautor. V prvej práci som testovala stabilné populácie *Procambarus virginalis* a *Faxonius limosus* na prítomnosť *A. astaci* v Bratislave na Slovensku v roku 2016. Prítomnosť patogénu račieho moru bola potvrdená iba u *F. limosus*; je však očakávaný horizontálny prenos *A. astaci* na *P. virginalis*, ako aj ďalšie šírenie tohto raka v rieke Dunaj. V druhej priloženej štúdii som testovala prítomnosť patogénu račieho moru u raka *Procambarus clarkii* v Indonézii, ako aj krabov *Parathelphusa convexa* a kreviet *Macrobranchium lanchesteri*, ktoré koexistujú v syntopii s jednou infikovanou populáciou tohto globálne najrozšírenejšieho invázneho raka. Prítomnosť *A. astaci* bola potvrdená u voľne žijúcej stabilnej populácie *P. clarkii* , ako aj z obchodu s domácimi zvieratami v Indonézii a u krabov a kreviet syntopických s *P. clarkii.* Zistenia poukazujú na hrozbu, ktorú šírenie sa tohto druhu predstavuje pre pôvodné raky v Indonézii a priľahlých regiónoch vrátane Novej Guiney a Austrálie.

Kľúčové slová: račí mor; *Aphanomyces astaci*; chronické infekcie; úhyny; genotyp; vektory

Abstract

Crayfish plague is an emerging disease caused by the oomycete *Aphanomyces astaci*, a pathogen listed among the 100 World´s Worst Invasive Alien Species. It was introduced into Europe in the second half of 19th century from North America and caused collapses of European native crayfish populations. Nowadays, *A. astaci* is widespread in Europe and has spread also to other parts of the world, threatening all susceptible crayfish of non-North American origin. The aims of this MSc thesis were 1) to provide information about crayfish plague outbreaks from recent years, and by using microsatellite and mtDNA markers reveal *A. astaci* genotypes involved; 2) to test healthy-looking indigenous crayfish for potential occurrence of chronic infections by *A. astaci* in Czechia. Six new crayfish plague outbreaks were confirmed from 2016 to 2018, involving at least five distinct pathogen strains. My results provide first evidence of the *A. astaci* genotype group D causing *Astacus astacus* and *Austropotamobius torrentium* mass mortalities in Czechia. MtDNA sequencing revealed two haplotypes of the D haplogroup, indicating two independent sources of infection presumably either from ornamental crayfish or spreading from neighbouring countries. The genotype group A was recorded in two *A. astacus* mortalities and genotype group E in one *A. torrentium* mortality. The genotype Up was re-identified in *A. astacus* outbreak after 13 years from its first occurrence in Czechia. In 15 tested populations of indigenous crayfish, no case was of chronic infection by *A. astaci* was observed. It seems that this phenomenon is not very common in Czechia; however, its occurrence cannot be ruled out. Apart from these results, summarized in the thesis in a form of a manuscript draft, I provide in appendices two published studies to which I contributed as a co-author. In the first, I have examined well-established populations of *Procambarus virginalis* and *Faxonius limosus* for the presence of *A. astaci* in Bratislava, Slovakia in 2016. The presence of the crayfish plague pathogen was confirmed only in *F. limosus*; however, horizontal transmission of *A. astaci* to *P. virginalis* is expected, as well as the further spreading of this crayfish in the Danube river. In the second attached study, I´ve tested for the presence of the crayfish plague pathogen *Procambarus clarkii* from Indonesia, as well as crabs *Parathelphusa convexa* and shrimps *Macrobranchium lanchesteri* coexisting in syntopy with one infected population of this globally most widespread invasive crayfish. The presence of *A. astaci* was confirmed in *P. clarkii* from outdoor established population as well as from pet trade in Indonesia, and in crabs and shrimps syntopic with *P. clarkii*. The findings highlight the threat the spread of this species present to native crayfish in Indonesia and adjacent regions, including New Guinea and Australia.

Key words: crayfish plague; *Aphanomyces astaci*; chronic infections; mortalities; genotype; vectors

1. Úvod

Sladkovodné raky sú najväčšími bezstavovcami v sladkovodnom prostredí. Ich veľkosť i funkcia ich činia kľúčovými pre stabilitu vodného ekosystému (Dorn a Wojdak 2004). Európske pôvodné sladkovodné druhy rakov zahŕňajú približne 15-16 druhov, niektoré však skôr tvoria druhové komplexy (Krandall a De Grave 2017; Pârvulescu 2019), z toho iba dva druhy sú pôvodné pre Českú republiku a to rak riavový (*Austropotamobius torrentium*) a rak riečny (*Astacus astacus*) (Kozák et al. 2014). Oba druhy sú považované za bioindikátory kvality vody (Kozák et al. 2014) a spolu s ostatnými druhmi rakov sú dôležitou súčasťou potravnej siete (Dorn a Wojdak 2004). Ich výskyt bol výrazne redukovaný v dôsledku chemického znečistenia vôd, konkurenčného vytlačenia zavlečenými druhmi rakov a v dôsledku šírenia račieho moru (Kozubíková a Petrusek 2009) (podrobnejšie v podkapitole 1.1).

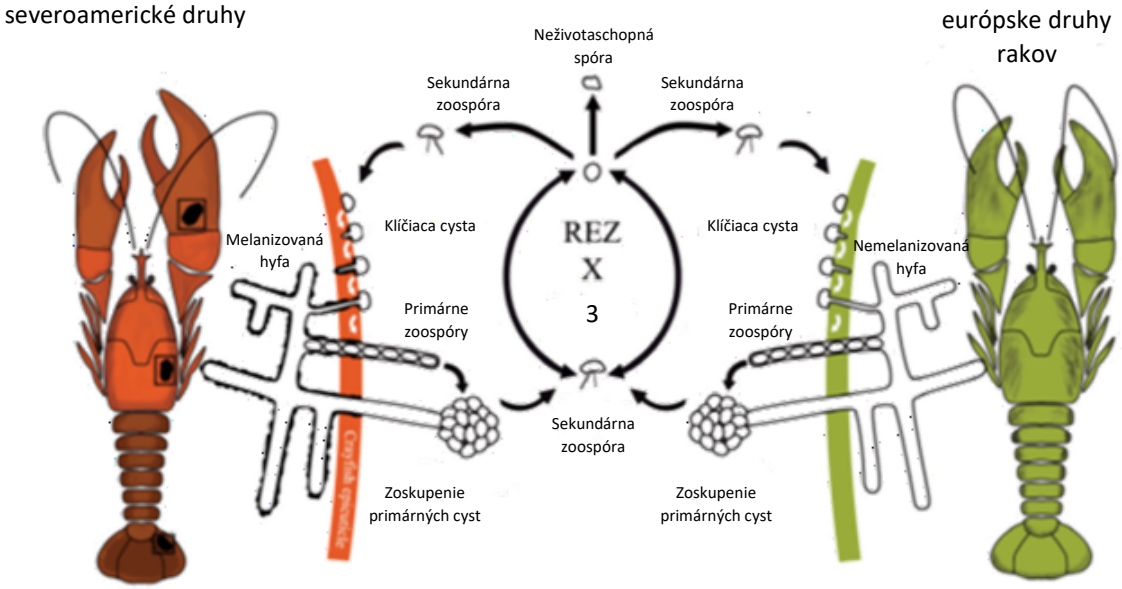
1.1 Račí mor

Tzv. „Emerging diseases" sú nové nákazy, ktoré sa rýchlo šíria alebo u patogénu došlo k zvýšeniu prevalencie či k rozšíreniu škály hostiteľov. Často sa jedná o patogén rozšírený v nedávnej dobe v dôsledku ľudských aktivít a takto nakazené živočíchy masívne hynú (Daszak et al. 2000). Medzi takéto ochorenia patrí aj račí mor.

Pôvodcom račieho moru je oomycéta *Aphanomyces astaci*, pôvodom zo Severnej Ameriky (Unestam 1972). Zdá sa, že dlhodobá koexistencia severoamerických rakov s *A. astaci* viedla ku spoločnej koevolúcii a severoamerické raky sa stali voči *A. astaci* odolnejšie (Söderhall and Cerenius 1999). Práve skrze nich sa dostal račí mor do Európy pravdepodobne v druhej polovici 19. storočia a jeho následne šírenie viedlo ku kolapsom populácii pôvodných európskych druhov rakov (Alderman 1996) Dnes je račí mor rozšírený v rôznych častiach Európy (zhrnuté v Svoboda et al. 2017), tak ako i v iných zákutiach sveta (Peiró et al. 2016, Mrugała et al. 2017; Martín-Torrijos et al. 2018, Putra et al. 2018) a ohrozuje zostávajúce populácie pôvodných naivných rakov (Holdich et al. 2009).

U *Aphanomyces astaci* je známy iba asexuálny spôsob rozmnožovania (Obr. 1), bez trvalého štádia (Söderhäll a Cerenius 1999; Alderman a Polglase 1986). Pôvodca račieho moru sa rozmnožuje pomocou zoospór, ktoré sú opatrené dvoma bočnými bičíkmi (Alderman a Polglase 1986), tieto zoospóry nešpecifickou chemotaxiou vyhľadávajú substrát na ktorom by mohli prosperovať, čo je povrch raka (Cerenius a Söderhäll 1984a). Po prisadnutí sa zo zoospóry encystáciou stáva cysta, ktorá klíči v hyfu, tá prerastá do kutikuly, u citlivejších druhov hlbšie do tkaniva a vytvára mycélium. Pokiaľ zoospóra prisadne na nevhodný podklad, encystácia sa dá zvrátiť a následne môže patogén ďalej vyhľadávať svojho hostiteľa (pro tento jav sa užíva anglický výraz „Repeated zoospore emergence" -

REZ), to sa môže opakovať aj trikrát (Cerenius and Söderhäll 1984b). Produkcia sporangii nastáva následne po kontaktu mycélia s vodou, vo vnútri nich sa tvoria primárne spóry, ktoré sa postupne uvoľňujú, hneď encystujú a vytvárajú primárne cysty, tieto cysty sa zoskupujú a vytvárajú tzv. „spore balls“. Životný cyklus je zakončený uvoľnením pohyblivých zoospór z primárnych cýst (Söderhäll a Cerenius 1999).



Obr. 1 Životný cyklus *Aphanomyces astaci* (upravené podľa Rezinciuc et al. 2015). (REZ - „repeated zoospore emergence“ - schopnosť raka zvrátiť encystáciu)

Raky infikované račím morom sa často chovajú veľmi netypicky, sú aktívne cez deň, často krát sa nachádzajú mimo svoje úkryty, taktiež sa môžu objaviť príznaky ako chôdza na vysoko zdvihnutých nohách, kŕčovité pohyby, ďalej strácajú koordináciu a únikový reflex (Alderman et al. 1984). Jedným z príznakov je tvorba melanínovych škvŕn (súčasť fenoloxidázovej kaskády), ku ktorej dochádza ak sa infekcia vyvíja dlhšie (Alderman et al. 1987), avšak prítomné melanínove škvrny nie je možné odlíšiť od melanínovych škvŕn spôsobených iným patogénom (Persson a Söderhäll 1983, podľa Kozubíková a Petrusek 2009) a zároveň nie vždy má infikovaný rak na sebe tieto škvrny, preto sa na základe ich výskytu nedajú robiť závery o prítomnosti infekcie (Kozubíková et al. 2009).

Pre druhy rodu *Aphanomyces* je typická vysoká hostiteľská špecifita (Diéguez-Uribeondo et al. 2009). Na základe toho, že v blízkosti ohnisiek račieho moru iné organizmy nevykazovali známky infekcie (Oidtmann 2012) a zároveň sa toto ochorenie nepotvrdilo u iných bentických kôrovcov ako sú napr. krivák (*Gammarus*) a žižavica (*Asellus*) (Kozubíková a Petrusek 2009; Svoboda et al. 2014a), bol predpoklad, že jediným hostiteľom sú raky. I keď v prvej polovici 20. storočia bol zaznamenaný prípad

o tom, že krab *Eriocheir sinensis* bol nakazený a uhynul na infekciu spôsobenú patogénom račieho moru (Benisch 1940, podľa Unestam 1972) a na základe toho bola vytvorená hypotéza, že *A. astaci* môže nakaziť aj iné desaťnožce (Decapoda) než raky (Unestam 1972). Avšak, až nedávno niekoľko štúdií túto hypotézu potvrdilo (Svoboda et al. 2014a,b; Schrimpf et al. 2014; Tilmans et al. 2014; Putra et al. 2018). Patogén račieho moru bol potvrdený u kraba *Eriocheir sinensis* vo Švédsku, kde koexistuje s *Pacifastacus leniusculus* (Svoboda 2014a), v Nemecku až na troch lokalitách v rieke Rýn so syntopickým výskytom s *F. limosus* a *F. immunis* (Schrimpf et al. 2014) a v Holandsku, kde sa syntopicky vyskytuje s *Faxonius limosus* (Tilmans et al. 2014). Ďalej bol tento patogén potvrdený v Turecku u kraba *Potamon potamios* koexistujúceho s *P. leptodactylus* (Svoboda et al. 2014a) a v Indonézii jak u kraba *Parathelphusa convexa*, tak u krevety *Macrobranchium lanchesteri* v syntopii s *Procambarus clarkii* (Putra et al. 2018). Experimentálne bola testovaná odolnosť kreviet *Neocardia davidi* a *Macrobrachium dayanum* voči *A. astaci*. Oba druhy kreviet sa zdajú byť odolné, avšak u niektorých jedincov *M. dayanum* bol zaznamenaný pravdepodobný rast *A. astaci* a jeho prítomnosť v niektorých jedincoch bola zachytená i po zvliekaní (Svoboda et al. 2014b). Navyše bola experimentálne potvrdená schopnosť prenosu *A. astaci* z kraba na raka (Schrimpf et al. 2014). Tieto desaťnožce síce nehynú v dôsledku nákazy, ale sú ďalším potencionálnym zdrojom šírenia infekcie pre vnímavé druhy rakov.

Patogén račieho moru prežíva výhradne v sladkovodnom prostredí (Unestam 1969) a jeho prenos je zabezpečený pohyblivými infekčnými zoospórami, ktoré sú buď voľne vo vode alebo v tkanive nakazeného živého i mŕtveho raka alebo iného desaťnožca či v jeho zvlečke (Oidtmann et al. 2002; zhrnuté v Svoboda et al. 2017). Uvoľnená spóra ma relatívne krátku životnosť a to niekoľko dní až týždňov, avšak v tele raka prežíva dokým je prítomný vhodný substrát (Unestam a Ajaxon 1978 podľa Kozubíková a Petrusek 2009). Priamy vertikálny prenos *A. astaci* na potomkov skrze vajíčka nebol zatiaľ preukázaný, avšak prenos z nakazenej samice na potomkov po ich vyliahnutí nemožno vylúčiť, keďže potomkovia sú až do prvého zvliekania prichytený na brušku samice (zhrnuté v Svoboda et al. 2017). Najbežnejší je však horizontálny prenos patogénu račieho moru (Svoboda et al. 2017), k tomu najviac prispela ľudská činnosť. Severoamerické raky boli do Európy pôvodne komerčne vyvážané pre spotrebné účely ľudí (Oidtmann et al. 2002), či na účel vysádzania namiesto pôvodných zdecimovaných európskych druhov (Holdich et al. 2006). Hlavne vysádzanie infikovaných severoamerických rakov viedlo k šíreniu patogénu na nové lokality (Nylund a Westman 1995). Dnes sa patogén šíri skôr cez zámerne vypustené infikované raky z akvaristických chovov (Chucholl 2013), či cez ornamentálne raky ktoré unikli z jazierok (Patoka et al. 2014). Spóry *A. astaci* sa pri akomkoľvek styku s vodou môžu preniesť, napr. cez rybárske náčinie či cez gumy na autách alebo cez zablatené gumáky (zhrnuté v Kozubíková a Petrusek 2009). K tomu napomáha taktiež zlá informovanosť verejnosti, kedy napr. rybári či potápači ani netušia či sa v danej oblasti vyskytuje račí mor a zároveň ani nevedia čo by mohli urobiť,

aby šíreniu mohli zabrániť (Kozubíková a Petrusek 2009) a tým môžu potencionálne prenášať patogén cez nedezinfikované náčinie.

Ďalšou možnosťou ako sa môže patogén preniesť ľudskou činnosťou je pri vysadzovaní rýb, kedy voda obsahuje zoospóry *A. astaci* (Nylund a Westman 1995). K šíreniu *A. astaci* však môžu napomôcť aj samotné ryby, bolo preukázané, že nedostatočne rozložená kutikula raka, ktorá prešla traktom rýb obsahovala hýfy *A. astaci*, ktoré úspešne infikovali rakov (Oidtmann et al. 2002).

Ďalej už je patogén šírený pomocou samotných infikovaných migrujúcich rakov (Oidtmann et al. 2002) či krabov (Svoboda et al. 2017). Donedávna sa myslelo, že šírenie račieho moru na dlhé vzdialenosti sa týka iba chronicky infikovaných severoamerických rakov (Svoboda et al. 2017) a bola zaužívaná predstava, že tam kde sa objaví račí mor, tak do niekoľkých dní populácia pôvodných európskych rakov skolabuje (Kozubíková et al. 2008), avšak s vývojom nových molekulárnych postupov začali pribúdať štúdie z rôznych európskych krajín o chronických infekciách račieho moru u pôvodných európskych druhov (zhrnuté Svoboda et al. 2017), čo poukazuje na to, že aj oni môžu byť relevantným zdrojom nákazy pre vnímavejšie populácie rakov. Problematiku prežívania hostiteľských populácii preberám detailnejšie vo svojej bakalárskej práci (M. Mojžišová 2016) a v úvode manuskriptu.

Pomocou metódy RAPD (Náhodná amplifikácia polymorfickej DNA) bolo pôvodne zistených päť genotypových skupín *A. astaci* (A, B, C, D, E) (Huang et al. 1994; Diéguez-Uribeondo et al. 1995; Kozubíková et al. 2011). Zdá sa, že genotypová skupina A pochádza z prvej introdukcie račieho moru do Európy (Huang et al. 1994). Niekoľko štúdii preukázalo jej nižšiu virulenciu oproti ostatným kmeňom *A. astaci* (Makkonen et al. 2012, 2014; Jussila et al. 2013; Viljamaa-Dirks et al. 2013, 2016; Becking et al. 2015). Práve znížená virulencia tohto kmeňa umožňuje existenciu chronických infekcii spôsobených *A. astaci* u pôvodných druhov, avšak môže sa jednať i o zvýšenú rezistenciu rakov, alebo kombináciu oboch možností (Jussila et al. 2014). Oboje naznačuje, že viac než storočná koexistencia tohto kmeňu *A. astaci* s vnímavými druhmi rakov viedla ku vzájomnej koevolúcii. Zvyšenú odolnosť rakov podporuje i existencia chronických infekcii spôsobená genotypovou skupinou B (Svoboda et al. 2014a; Maguire et al. 2016; Kokko et al. 2018; Panteleit et al. 2018), ktorá je virulentnejšia než genotypová skupina A (Makkonen et al. 2012, 2014; Jussila et al. 2013; Viljamaa-Dirks et al. 2013, 2016; Becking et al. 2015). Zatiaľ, čo chronické infekcie spôsobené genotypovou skupinou A boli dokumentované u *A. astacus*, *A. torrentium*, *A. pallipes* a *P. leptodactylus* (zhrnuté v Svoboda et al. 2017; Kokko et al. 2018), genotypová skupina B bola dokumentovaná iba u *P. leptodactylus* (Svoboda et al. 2014a; Kokko et al. 2018; Panteleit et al. 2018). Vyššia odolnosť tohto druhu bola preukázaná aj v štúdii Unestam (1969).

Račí mor sa na územie Česka dostal koncom 19. storočia, avšak tomuto ochoreniu sa v Česku v 20. storočí nevenovalo príliš pozornosti a neboli dostupné ani vhodné diagnostické metódy a preto máme chabé poznatky z tohto obdobia (Kozubíková et al. 2006). Až s rozvojom diagnostických metód sa v posledných dvoch desaťročiach podarilo dokumentovať niekoľko úhynov na račí mor na území

Česka, tieto úhyny sú detailne zhrnuté v prácach Kozubíková et al. (2006, 2008) a Kozubíková-Balcárová et al. (2014). Na území Česka bolo celkom medzi rokmi 2004 až 2014 zaznamenaných 11 masových úhynov pôvodných rakov v dôsledku račieho moru, avšak úhynov bolo pravdepodobne viac a toto je len špička ľadovca (Kozubíková et al. 2008; Kozubíková-Balcárová et al. 2014). Za úhyny boli zodpovedné genotypové skupiny patogénu A, B, E a neobvyklý genotyp Up (Grandjean et al. 2014). Iba v dvoch prípadoch bol odhalený pravdepodobný lokálny zdroj infekcie a to u genotypovej skupiny E (Kozubíková-Balcárová et al. 2014). U ostatných genotypových skupín a genotypu Up bol zdroj infekcie nejasný, keďže v bezprostrednej blízkosti sa nenachádzali žiadne severoamerické raky.

Na základe nejasných zdrojov zdokumentovaných úhynov a reportov o výskyte chronických infekcii u pôvodných druhov zo zahraničia, bola v štúdii Kozubíková-Balcárová et al. (2014) vytvorená hypotéza, že zdrojom infekcie úhynov v ČR môžu byť chronicky infikované pôvodné druhy rakov, avšak doteraz ju nikto neotestoval.

1.2 Ciele práce

Cieľom mojej diplomovej práce bolo otestovať hypotézu, že by sa chronické infekcie račieho moru u pôvodných druhov mohli vyskytovať aj na území Česka (Kozubíková-Balcárová et al. 2014). Avšak výsledky nepotvrdili túto hypotézu, takže som sa viac zamerala na druhý cieľ práce a to bolo testovať variabilitu patogénu račieho moru v nedávno doložených masových úhynoch pôvodných rakov na území Česka.

Popri tom som ešte spolupracovala na iných projektoch zameraných na račí mor, ktoré boli publikované. Tieto publikácie prikladám ako prílohu 1 (Lipták et al. 2017) a Prílohu 2 (Putra et al. 2018). V práci Lipták et al. (2017) bolo mojím cieľom otestovať na prítomnosť patogénu račieho moru populácie rakov *P. virginalis* a *F. limosus*. V práci Putra et al. (2018) bolo mojím cieľom otestovať na prítomnosť *A. astaci* raka *P. clarkii* jak z akvaristických chovov, tak voľne žijúcu populáciu a taktiež kraby *Parathelphusa convexa* a krevety *Macrobranchium lanchesteri* v syntopii s týmto druhom raka.

2. Zhrnutie nasledujúceho rukopisu a publikovaných prác

Celkovo som otestovala 15 populácií zdravo vyzerajúcich jedincov *A. astacus* a *A. torrentium*, avšak v ani jednej populácii som nezaznamenala prítomnosť chronickej infekcie. Tento fenomén sa zdá že na území Česka nie je bežný, avšak na základe mojich výsledkov ho nemôžeme vylúčiť. Keďže sa jednalo o zákonom chránené druhy, testovali sme iba vybrané populácie, snažili sme sa zamerať na tie, kde už bol račí mor v minulosti dokumentovaný. Ďalej sme netestovali populácie *P. leptodactylus,* ktoré

sa zdajú byť ako sa ukazuje až v posledných rokoch častým nositeľom chronických infekcii račieho moru v iných krajinách (Svoboda et al. 2014a; Maguire et al. 2016; Kokko et al. 2018; Panteleit et al. 2018). Práve z toho dôvodu by malo byť ďalšie testovanie zamerané na populácie *P. leptodactylus* na území Česka.

Ďalej som popri tom testovala masové úhyny pôvodných druhov rakov, z ktorých výsledky sa stali nakoniec základom mojej diplomovej práce. Behom dvoch rokov 2016 - 2018 som zdokumentovala šesť úhynov na račí mor u *A. astacus* a *A. torrentium* a pomocou mikrosatelitových a mtDNA markerov som určila jednotlivé genotypy patogénu račieho moru. Táto práca poskytuje prvý dôkaz o prítomnosti genotypovej skupiny D na území Česka, a naviac pomocou sekvenovania ribozomálných podjednotiek mitochondriálnej DNA som identifikovala dva rôzne haplotypy z D haplotypovej skupiny (d1, d2), to naznačuje nezávisle zdroje infekcie. Ďalej som identifikovala genotypové skupiny A a E a genotyp Up, ktorý bol v Česku zaznamenaný iba raz v rieke Úpoř v roku 2005. Podobne ako v práci Kozubíková-Balcárová et al. (2014) zdroj infekcie bol určený iba pre úhyn genotypovou skupinou E a u ostatných úhynov bol zdroj infekcie nejasný. Možné zdroje infekcie rozoberám následne v diskusii manuskriptu.

Ďalej prikladám k manuskriptu, dve prílohy, kde som prispela ako spoluautor počas mojich štúdii na magisterskom stupni.

V prvej priloženej práci (Lipták et al. 2017) som testovala populácie rakov *Procambarus virginalis* a *Faxonius limosus* na prítomnosť patogénu račieho moru v rieke Dunaj na Slovenskom území v roku 2016. Oba druhy sú typickými prenášačmi račieho moru (Keller et al. 2014; Mrugała et al. 2015). Zatiaľ čo testovaná populácia *F. limosus* sa do Dunaja v Bratislave dostala pravdepodobne aktívnym šírením z Maďarska (Lipták a Vitázková 2014), populácia *P. virginalis* má pravdepodobný pôvod z akvaristických chovov (Chucholl et al. 2012). Jeden jedinec tohto druhu stačí, aby dal vzniknúť novej populácii, a to vďaka jeho partenogenetickému rozmnožovaniu (Martin et al. 2010). Oba testované druhy sú v bezprostrednej blízkosti avšak bariéru im tvorí čerpacia stanica (Lipták et al. 2017). V tejto štúdii som potvrdila prítomnosť patogénu račieho moru iba v populácii *F. limosus*, avšak do budúcna sa očakáva že dôjde k horizontálnemu prenosu patogénu na *P. virginalis* a spolu s ním k ďalšiemu šíreniu račieho moru proti prúdu rieky Dunaj (Lipták et al. 2017). I keď sme v tejto štúdii nepotvrdili prítomnosť patogénu račieho moru u *P. virginalis* (Lipták et al. 2017), nemôžme vylúčiť, že takto nakazené populácie sa na Slovensku nevyskytujú. U *P. virginalis* , v akvaristických chovoch aj voľne v prírode bola potvrdená genotypová skupina D, preto by sa mala zvážiť možnosť, že takto nakazený *P. virginalis* by sa mohol vyskytovať aj na Slovensku a ďalej šíriť do Česka.

V druhej priloženej práci (Putra et al. 2018) som testovala na prítomnosť patogénu račieho moru populácie rakov *Procambarus clarkii* v Indonézii, tak ako aj krabov *Parathelphusa convexa* a kreviet *Macrobranchium lanchesteri*, ktoré s týmito rakmi koexistujú. Račí mor sa okrem Európy (Holdich et

al. 2009) rozšíril aj do iných častí sveta, nedávno bol jeho výskyt potvrdený v Južnej Amerike a v Japonsku (Peiró et al. 2016; Mrugała et al. 2017; Martín-Torrijos et al. 2018). V oboch oblastiach sa šírenie račieho moru spája s výskytom raka *Procambarus clarkii*, u ktorého bola infekcia potvrdená. Tento rak sa šíri vďaka importom či už pre akvaristické účely alebo pre konzumáciu (Souty-Grosset et al. 2016). V tejto štúdii som potvrdila prítomnosť patogénu račieho moru u *P. clarkii* jak v lokálnom obchode so zvieratami, tak z voľne žijúcej populácie. Navyše som potvrdila prítomnosť *A. astaci* aj u testovaných krabov a kreviet, čo zdôrazňuje hrozbu, ktorú šírenie tohto raka predstavuje pre najbližšie oblasti ako sú Nová Guinea alebo Austrália (Putra et al. 2018). V Česku bol *P. clarkii* zatiaľ dokumentovaný iba v akvaristických chovoch, avšak bola uňho potvrdená infekcia spôsobená *A. astaci* a navyše genotypovou skupinou D (Mrugała et al. 2015). Vypustenie takto infikovaných jedincov, môže mať veľmi negatívny dopad. Obe priložené štúdie sú zamerané na testovanie prítomnosti patogénu račieho moru u invazívnych rakov rozšírených do nových lokalít, s najväčšou pravdepodobnosťou v dôsledku ich vypúšťania chovateľmi do voľnej prírody či ich úniku zo záhradných jazierok. Je pravdepodobné, že tieto druhy rakov, by mohli byť zdrojom infekcie spôsobenej genotypovou skupinou D, ktorú som dokumentovala v mojej štúdii.

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Crayfish plague in Czechia: new outbreaks and testing for chronic infections

(manuscript draft, only written by MM with supervisor's contribution of AP; feedback of other co-authors yet to be incorporated)

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Abstract

The crayfish plague pathogen *Aphanomyces astaci* belongs to the most studied invertebrate pathogens, which co-evolved with American crayfish species and causes mass mortalities of European and Asian crayfish species. In this study we document six crayfish plague outbreaks that occurred from 2016 to 2018 in Czechia, and by using available molecular techniques (microsatellite and mtDNA markers) we reveal *A. astaci* genotypes involved. Our results provide first evidence of the genotype group D causing *A. astacus* and *A. torrentium* mass mortalities in Czechia. Moreover, mtDNA sequencing confirmed two distinct haplotypes of the D haplogroup (d1 and d2), indicating two independent sources of infection, presumably either from ornamental crayfish or spreading from neighbouring countries. The genotype group A was recorded in two *A. astacus* mortalities and genotype group E in one *A. torrentium* mortality. Microsatellite genotyping reidentified the genotype Up in *A. astacus* outbreak after 13 years from its first occurrence. In addition, we tested healthy-looking indigenous crayfish from 15 populations for potential presence of chronic infections by *A. astaci* in Czechia. We did not reveal any such case; thus, it seems that chronic infections by *A. astaci* are not common in Czechia. However, their presence in the country, especially in *Pontastacus leptodactylus* populations introduced to the country since the late 19th century, cannot be excluded.

Key words: *Aphanomyces astaci*; native crayfish; invasive crayfish; microsatellite genotyping; mitochondrial markers, mass mortalities

1. Introduction

The crayfish plague pathogen, the oomycete *Aphanomyces astaci*, originally from North America, was introduced into Europe in the second half of the 19th century and has spread since then into most of the continent (Alderman 1996). Expansion of *A. astaci* caused collapses of the many native European crayfish populations and in the present still pose a threat for those remaining in Europe (Holdich et al. 2009) and also for crayfish from other parts of the world (Peiró et al. 2016, Martín-Torrijos et al. 2018, Putra et al. 2018). Because of its virulence and strong devastating effect on the European native crayfish species, *A. astaci* was classified among the world´s 100 worst invasive alien species (Lowe et al. 2004). The original natural hosts of *A. astaci* are the North American crayfish species (Söderhall and Cerenius 1999), which are considered more resistant against *A. astaci* than the native European crayfish (Cerenius et al. 2003; reviewed in Svoboda et al. 2017). Presumably, the common history of the North American crayfish species with *A. astaci* led to an effective host immune response, so the populations of these crayfish serve as long-term reservoirs of the pathogen (Söderhall and Cerenius 1999).

Originally, five *A. astaci* genotype groups have been defined by Random Amplified Polymorphic DNA (RAPD), labelled alphabetically from A to E (for more details about the nomenclature, see Svoboda et al. 2017). The genotype group A probably comes from the first wave of *A. astaci* invasion; it was originally isolated from *Astacus astacus* and *Pontastacus leptodactylus*, but its original American host is still unknown (Huang et al. 1994). The genotype groups B and C were isolated from *Pacifastacus leniusculus* (Huang et al. 1994). Whereas the genotype group B was involved in many crayfish plague outbreaks across Europe (Svoboda et al. 2017), the presence of the genotype group C has not been documented in Europe so far. The genotype group D was isolated originally from *Procambarus clarkii* (Diéguez-Uribeondo et al. 1995) and the genotype group E from *Faxonius limosus* (Kozubíková et al. 2011a).

The application of recently developed microsatellite markers (Grandjean et al. 2014) and primers targeting mitochondrial small and large ribosomal subunits (rnnS and rnnL; Makkonen et al. 2018) provides a better insight into the identity and origin of individual pathogen strains. The microsatellite genotyping revealed greater variability within RAPD-defined *A. astaci* genotype groups and allows identification of yet unknown *A. astaci* strains (Grandjean et al. 2014, James et al. 2017, Mrugała et al. 2017, Caprioli et al. 2018, Panteleit et al. 2019).

The mtDNA markers separated RAPD-genotype groups into four haplogroups (A, B, D, E) and revealed their correspondence to the two main mitochondrial lineages, one consisting of A, B and E haplogroups, and the other one of the D haplogroup (Makkonen et al. 2018). The A-haplogroup contains the RAPD-defined genotype groups A and C, and haplogroups B, D and E are assigned with

the genotype groups labelled by the same letters. Within the D-haplogroup, three closely related haplotypes differing in 1-4 of nucleotide positions were documented so far (Makkonen et al. 2018; Martín-Torrijos et al. 2018).

Moreover, an unusual genotype "Up", only known from crayfish hosts (Grandjean et al. 2014; Panteleit et al. 2018), was considered as closely related to the genotype group B based on similarity of microsatellite loci (Kozubíková-Balcarová et al. 2014; Grandjean et al. 2014), however mtDNA sequencing linked this genotype to the A haplogroup (Makkonen et al. 2018; Panteleit et al. 2018). This is also the case of the strain identified in *Faxonius rusticus* in North America (Panteleit et al. 2019). The Up genotype was originally identified in *A. torrentium* mass mortality in Czechia (Grandjean et al. 2014), however it also was identified in chronically infected *P. leptodactylus* population from Romania (Panteleit et al. 2018).

Most European crayfish species are highly susceptible to crayfish plague (Alderman et al. 1987) and after infection by this disease usually die quickly (Unestam 1969). However, several field-based studies recently documented chronic infections not associated with mass mortalities (reviewed in Svoboda et al. 2017), or slowly developing crayfish plague outbreaks (Viljamaa-Dirks et al. 2011; Caprioli et al. 2013).

Most early studies confirming chronic infections by molecular detection of *A. astaci* did not genotype the pathogen (Svoboda et al. 2012; Kokko et al. 2012; Pârvulescu et al. 2012; Schrimpf et al. 2012; Kušar et al. 2013), but later studies did so (Svoboda et al. 2014a; Kokko et al. 2018; Panteleit et al. 2018; Jussila et al. 2017). A notable exception were the early studies from Finland, where the performing of RAPD genotyping was possible thanks to isolation of pure cultures of *A. astaci* strains (Jussila et al. 2011; Viljamaa-Dirks et al. 2011,2013). The occurrence of chronic infections, in which the genotype group A was identified, has been documented in some populations of *Astacus astacus* in Finland (Jussila et al. 2011; Viljamaa-Dirks et al. 2011,2013) and in Croatia (Maguire et al. 2016), *Austropotamobius pallipes* in Italy (Manfrin and Pretto 2014), *A. torrentium* in Croatia (Maguire et al. 2016) and Slovenia (Jussila et al. 2017) and in some populations of *Pontastacus leptodactylus* in Croatia (Maguire et al. 2016) and Turkey (Kokko et al. 2018). Moreover, chronic infections by the genotype group B have also been reported in some populations of *P. leptodactylus* in Turkey (Svoboda et al. 2014a; Kokko et al. 2018), Croatia (Maguire et al. 2016), and Romania (Panteleit et al. 2018).

The presence of chronic infection might be a consequence of resistance of the host, reduced virulence of the pathogen, or both (Jussila et al. 2014). In at least some cases, virulence of the strain isolated from chronic infection was confirmed experimentally (Mrugała et al. 2016; Jussila et al. 2017) which may indicate coevolution of *A. astaci* with its crayfish hosts. This is also supported by other several experimental studies (Makkonen et al. 2012, 2014; Jussila et al. 2013; Viljamaa-Dirks et al. 2013, 2016; Becking et al. 2015), which revealed reduced virulence of the pathogen as well as

variability in resistance in some indigenous crayfish populations (Jussila 2011; Makkonen et al. 2012, 2014; Martín-Torrijos et al. 2017).

There are thus several sources from which native European crayfish may become infected by crayfish plague. The most frequent pathway of infection is through the direct contact with invasive North American crayfish species, as well as by zoospores long-distance spread downstream from non-indigenous crayfish or from indigenous crayfish with acute or chronic infection (reviewed in Svoboda et al. 2017). Further sources of crayfish plague may be infected non-indigenous crayfish intentionally released from aquaria or escaped from garden ponds (Patoka et al. 2014); even if these individuals are not established, they may serve as vectors of *A. astaci* (Patoka et al. 2018). Moreover, in some parts of the world, other freshwater decapod crustaceans (Schrimpf et al. 2014; Tilmans et al. 2014; Svoboda et al. 2014a,b; Putra et al. 2018) may serve as a reservoir of crayfish plague.

In Central and Western Europe, there are four recognized native crayfish species, of which two (*A. astacus* and *A. torrentium*) are native to Czechia (Kozák et al. 2014). An additional one (*P. leptodactylus*) was introduced in the second half of 19th century and became a part of Czech fauna (Horká 2006). Now, this species is considered as naturalised, however there are some speculations that *P. leptodactylus* may be native to the southernmost part of the Morava river basin (Jurek and Chytrý 2015). . In contrast, the number of non-indigenous crayfish species occurring In Central and Western Europe is much higher (Kouba et al. 2014; Weiperth 2017, 2019). The most harmful are rapidly spreading invasive crayfish species from North America, which threaten native crayfish species by their higher competitive ability (Lindquist and Huner 1999) as well as by transmission of crayfish plague (Holdich et al.2009).Two of them have spread in Czechia, i.e. *Faxonius limosus, Pacifastacus leniusculus* (Petrusek et al. 2006; Filipová et al. 2006a, b). Chronic infections by *A. astaci* have been widely documented in these two non-indigenous crayfish species across Czechia (Kozubíková et al. 2009, 2011a); however, no traces of the pathogen , even at the lowest level, were detected in some populations (Kozubíková et al. 2009, 2011a; data of M. Mojžišová in Hladovec 2017). Moreover, *Procambarus virginalis* has been documented in two water bodies of Czechia, without evidence of infection by *A. astaci* (Patoka et al. 2016).

The first reports about crayfish plague at the territory of Czechia are from the late 19th century (Kozubíková et al. 2006). After decades of low interest in this topic (and insufficient diagnostic tools throughout the 20th century), the other confirmed cases of the crayfish plague outbreaks are known since 2004 (Kozubíková et al. 2006). Altogether, 11 crayfish plague outbreaks were thoroughly documented between 2004 and 2014 in Czechia (Kozubíková et al. 2008, Kozubíková-Balcarová et al. 2014), with confirmed genotype groups A, B, E and the genotype Up (Grandjean et al. 2014) (Fig. 1).

The aim of this study is to provide up-to-date information on crayfish plague outbreaks in Czechia and elucidate which genotype strains are responsible for the most recent outbreaks by using available

molecular techniques including microsatellite and mtDNA markers. In addition, we examined healthy-looking *Astacus astacus* and *Austropotamobius torrentium* populations for potential occurrence of chronic infections caused by the crayfish plague pathogen. Their occurrence in native crayfish populations has been already documented from several European countries (reviewed in Svoboda et al. 2017) and moreover, in Croatia such phenomenon seems to be common (Maguire et al. 2016). Thus, we hypothesized that chronic infections caused by *A. astaci* might occur in some native crayfish populations in Czechia (and serve as source of at least the genotype group A). This was already speculated about by Kozubíková-Balcarová et al. (2014) but no data from Czechia are available.

2. Materials and methods

2. 1. Crayfish samples

Specimens of native European crayfish species, the noble crayfish *Astacus astacus* and the stone crayfish *Austropotamobius torrentium*, were obtained from 10 Czech localities where crayfish mortalities were reported (Table 1, Fig. 1). One of these mortalities, which affected syntopic populations of *A. astacus* and *A. torrentium* in three connected brooks (Kublovský, Stroupinský and Bzovský), was considered in our study as a single locality. The collection of whole bodies of freshly dead individuals was performed manually by relevant nature conservation authorities and obtained specimens were stored in bottles with 96 % ethanol. In some cases, the specimens were placed into separate plastic bags and cooled on ice in transport box, and subsequently stored in the freezer at -80 °C until further manipulation.

Four to ten individuals were processed from eight *A. astacus* populations and from three *A. torrentium* populations (Table 1). The number of tested individuals per population varied depending on the infection status; when crayfish plague was confirmed, no more samples were analysed. The soft abdominal cuticle, soft parts of joints and melanized spots, if present, were dissected, to increase probability of successful detection of the crayfish plague pathogen (Oidtmann et al. 2006). Dissected tissue was placed into 1.5 ml Eppendorf tubes and stored in 96% ethanol until further processing.

In addition, together with co-workers with appropriate permission, tissues from 175 healthy-looking individuals from 15 native crayfish populations (10 populations of *Astacus astacus* and 5 populations of *Austropotamobius torrentium*) were sampled from manually caught specimens (Table 2, Fig. 1). Out of these, three populations of *A. astacus* and one population of *A. torrentium* were sampled on localities with documented previous crayfish plague episodes, and three populations of *A.*

Table 1. Summary of crayfish plague outbreaks and other crayfish mortalities documented in Czechia in 2016-2018. All crayfish plague outbreaks were successfully genotyped at microsatellite loci (Grandjean et al. 2014). Sequences of *A. astaci* mtDNA markers (Makkonen et al. 2018) were obtained for all but one outbreak (Ostrovský brook), from which no DNA isolates were available for mtDNA sequencing. DNA isolates with the highest agent levels were used for the genotyping. N/A: not available

Species	Locality (Administrative region)	Watershed	Geographical coordinates	Year	N analy-sed	Agent level	N geno-typed	SSR	mtDNA haplo-type
Crayfish plague outbreaks									
A. astacus	Ostrovský brook (Central Bohemia)	Sázava	49.7658°N, 15.2082°E	2016	4	A3-A5	2	SSR-A1	N/A
A. astacus	Husinec dam (South Bohemia)	Blanice	49.0364°N, 13.9834°E	2018	9	A3-A7	2	SSR-A1	a
A. astacus	Klenský brook (South Bohemia)	Vltava	48.7813°N, 14.6199°E	2018	10	A3-A6	2	SSR-UP	a
A. astacus	river Rožnovská Bečva (Zlín)	Morava	49.4570°N, 18.1340°E	2018	5	A4-A6	3	SSR-D	d2
A. astacus A. torrentium	Stroupinský, Bzovský, Kublovský brooks (Central Bohemia)	Berounka	49.8967°N, 13.8900°E	2018	9 9	A4-A7	3 3	SSR-D	d1
A. torrentium	Radotínský brook (Central Bohemia)	Berounka	49.9985°N, 14.3181°E	2017	4	A4-A7	2	SSR-E	e
Other crayfish mortalities									
A. astacus	Pelechovský pond (Pardubice)	Elbe	49.9926°N, 15.5512°E	2018	4	A0			
A. astacus	Javornický brook (Hradec Králové)	Elbe	50.1697°N, 16.3028°E	2018	9	A0			
A. astacus	River Moravice (Moravia-Silesia)	Oder	49.8544°N, 17.8495°E	2018	4	A0			
A. torrentium	river Bradava (Plzeň)	Berounka	49.6176°N, 13.5749°E	2016 2017	10 4	A0			

astacus and two populations of *A. torrentium* were sampled in the brooks near known historical outbreaks, one sampled population of *A. astacus* was syntopic with *Pacifastacus leniusculus,* and two were in brooks close to *P. leniusculus* populations. To minimize negative effect of sampling on specimens of protected species, only one pereopod and one uropod were always taken from living crayfish, which were then released back to the locality. The tissues were placed into small vials and fixed by 96% ethanol.

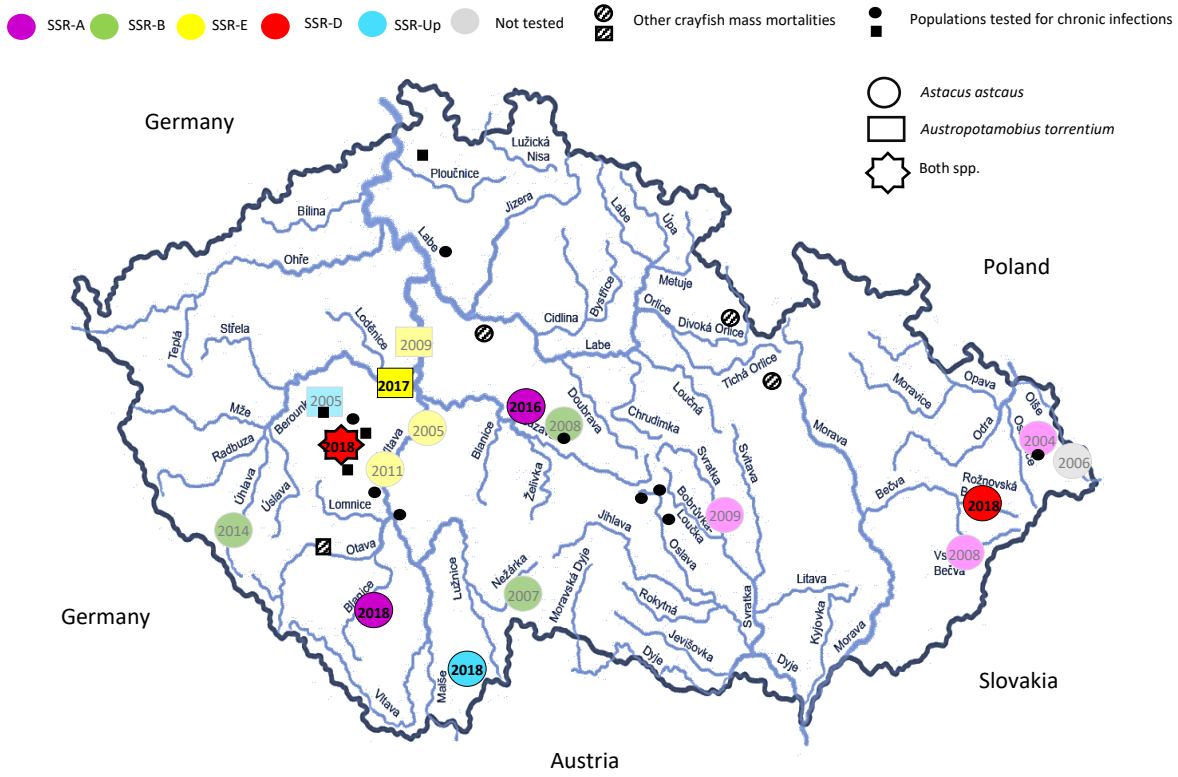

Fig. 1 Distribution of crayfish plague outbreaks affecting *Astacus astacus* and *Austropotamobius torrentium* populations in Czechia, documented between 2004 and 2018, and location of crayfish populations tested for chronic infections. Crayfish species are indicated by symbol shape (circles vs. rectangles), *A. astaci* genotypes (as defined by microsatellite markers) detected in outbreaks by different colours. Newly reported crayfish plague outbreaks from 2016 to 2018 have bright-coloured symbols, older outbreaks from 2004 to 2014, reported in Kozubíková et al. (2008) and Kozubíková-Balcarová et al. (2014) are in paler shades of the same colours. Small striped symbols indicate other mass mortalities of both indigenous crayfish species from 2016 to 2018 and small black symbols indicate populations of both indigenous crayfish species sampled from 2013 to 2017 for testing for occurrence of *A. astaci* chronic infections.

Table 2. Summary of the sampling sites and indigenous crayfish individuals tested for potential occurrence of chronic infections in Czechia from 2013 to 2017. No analysed individual was *A. astaci*-positive. Localities with documented previous crayfish plague outbreaks are marked with an asterisk. N: the number of analysed crayfish individuals

Locality (Administrative region)	Watershed	Geographical coordinates	Year	n
Astacus astacus				
Žebrákovský brook (Vysočina) *	Sázava	49.6939°N, 15.3848°E	2013	10
Losinský brook (Central Bohemia)	Sázava	49.8271°N, 15.0882°E	2013	11
brook Pšovka (Central Bohemia) *	Elbe	50.4130°N, 14.5768°E	2013 2015	21 9
brook Křivec (Moravia-Silesia) *	Oder	49.6816°N, 18.6653°E	2013	10
Ohrazenický brook (Central Bohemia)	Berounka	49.7887°N, 13.9627°E	2015	33
Červený brook (Central Bohemia)	Berounka	49.7883°N, 13.8820°E	2015	5
Balinka brook (Vysočina)	Morava	49.3459°N, 15.9426°E	2017	4
Zátoky brook (Vysočina)	Morava	49.4273°N, 15.9743°E	2017	4
river Oslava (Vysočina)	Morava	49.4207°N, 15.9858°E	2017	3
Austropotamobius torrentium				
Úpořský brook (Central Bohemia) *	Berounka	49.9667°N, 13.8596°E	2013	8
Bzovský brook (Central Bohemia)	Berounka	49.8967°N, 13.8830°E	2015	24
Prešínský brook (Plzeň)	Berounka	49.5621°N, 13.6131°E	2015	3
Huníkovský brook (Ústí and Labem)	Elbe	50.7775°N, 14.4212°E	2015	7
Syntopic population of A. torrentium and A. astacus				
Kublovský brook (Central Bohemia)	Berounka	49.9053°N, 13.8987°E	2015	22 1

2. 2. Molecular detection of Aphanomyces astaci

2. 2. 1. Extraction of DNA

The DNA extraction followed established protocols, as described in Oidtmann et al. (2006) and Kozubíková et al. (2008). In short, the obtained tissue was ground in liquid nitrogen and subsequently, DNA extraction was performed with the DNeasy tissue kit (QIAGEN) from up to 50 mg of homogenised mixture according to manufacturer´s protocol.

In every DNA isolation batch, two negative controls, consisting of Eppendorf tubes containing 50 µl of sterile deionized water, were included to check for possible contamination during the isolation process. An "environmental control" was kept opened during the grinding step, a "DNA extraction control" was kept closed. In the subsequent extraction using the DNeasy tissue kit, the negative controls were treated like DNA samples. In all cases, no traces of *A. astaci* DNA was detected in these controls.

2. 2. 2. Quantitative real-time PCR

TaqMan Minor Groove Binder (MGB), quantitative PCR (qPCR) assay according to Vrålstad et al. (2009) with minor modifications was used for detection of *A. astaci* DNA on the Bio-Rad iCycler iQ5 thermal cycler. The PCR reactions were carried out in 25 µl volumes, consisting of 20 µl of reaction mix and 5 µl of DNA isolate. The mix contained 1.5 µl of sterile Milli-Q water, 2.5 µl (5µM) of *A. astaci*-specific primers (AphAstITS-39F and AphAstITS-97R), 1 µl (5µM) of MGB fluorescent probe (AphaAstITS-60P, FAM; Applied Biosystems) and 12.5 µl of *Taq Man environmental Master Mix 2.0* (Applied Biosystems). The PCR program consisted of 2 min at 50 °C, 10 min at 95 °C, and 50 cycles of 15 s at 95°C, 30 s at 62 °C (Svoboda et al. 2014a). Each run contained additional "no template" control, consisting solely of 20 µl of the reaction mix, to reveal possible false positive results due to reagent contamination.

Four calibrants (standards of *A. astaci* DNA of known concentration) were used for quantification of pathogen DNA in PCR-forming units (PFU) in the reaction (according to Vrålstad et al. 2009). Results were evaluated using iQ5 Optical System Software (version 2.0, Bio-Rad).

As inhibition of PCR reaction may influence the results, apart from the use of Taq Man Environmental Master Mix 2.0 that was reported to minimize inhibition (Strand et al. 2011), selected DNA isolates were tested twice, undiluted and 10-fold diluted (Vrålstad et al. 2009, Kozubíková et al. 2011b, Svoboda et al. 2014a). These included all isolates from crayfish mortalities, and ca 25% of DNA isolates from healthy-looking individuals, selected randomly in every PCR batch. The eventual inhibition of PCR reaction would be revealed when comparing the cycle threshold (Ct) values between the undiluted and corresponding 10-fold diluted isolates; theoretical Ct value difference is 3.32, but variation of up to 15 % was considered acceptable (as in Kozubíková et al. 2011b). No inhibition was observed in any samples for which dilutions were performed.

If the PFU value from diluted DNA isolate was below 50 (the limit of quantification), the final PFU value was taken from undiluted isolate only. If the PFU values from the dilution exceeded 50, the final PFU value was calculated as a mean of the value from undiluted and ten times multiplied value of diluted isolate (Kozubíková et al. 2011b). Based on the PFU values, each sample was assigned to semi-

quantitative agent levels (Vrålstad et al. 2009, Kozubíková et al. 2011b), ranging from A0 (no pathogen DNA detected) to A7 (exceptional high levels of pathogen DNA in the sample) (Table 3). Samples with agent levels A2 or higher were considered *A. astaci*-positive.

2. 3. Microsatellite genotyping of A. astaci

Nine microsatellite markers were used to determine *A. astaci* genotype groups involved in the crayfish plague outbreaks as described in Grandjean et al. (2014). A minimum level of *A. astaci* DNA in the isolate corresponding to ca 900 - 1000 PFU (level A3 - A4) is required to successfully amplify individual loci (Grandjean et al. 2014). All samples from outbreaks exceeded this threshold. As the result from marker multiplexing were not consistent, the amplification was done separately for each locus.

Each reaction consisted of 1 µl of DNA isolate, 0.5 µl of both reverse and forward primers (10 µM; one of the labelled by fluorescent dye) for each locus (Aast2, Aast4, Aast6, Aast7, Aast9, Aast10, Aast12, Aast13, Aast14), 5 µl of QIAGEN multiplex master mix (part of the QIAGEN Multiplex PCR Kit), 1 µl of QIAGEN's Q-solution and 2 µl of RNase-free water to reach the final volume 10 µl in 0.2 ml tube. The PCR program consisted of 15 min at 95 °C, 40 cycles of 30 s at 95 °C, 90 s at 54 °C, 60 s at 72°C, and final step of 10 min at 72 °C (Grandjean et al. 2014). The amplification success was checked by agarose electrophoresis. Each PCR product (0.5 µl) was mixed with 9.25 µl of formamide and 0.25 µl of standard (The GeneScan™ 500 LIZ® Size Standard) and denatured by heating to 95 °C for 2 min, and sent for fragment analysis to the DNA Sequencing Laboratory of the Faculty of Science, Charles University. The allele sizes at each locus were scored using the GeneMarker software (version 1.95, SoftGenetics LLC) and compared with multilocus genotypes of *A. astaci* reference strains (Table 3).

2. 4. Sequencing of A. astaci mtDNA

Fragments of two mitochondrial genes for ribosomal rnnS and rnnL subunits (512 bp and 435 bp, respectively) were amplified and sequenced to verify results from the microsatellite genotyping for samples from all but one confirmed outbreak (the exception being Ostrovský brook), and to obtain better insight into variability of *A. astaci* strains that caused the outbreaks. The protocol followed in general Makkonen et al. (2018). The PCR reactions were carried out in 25 µl volumes containing 12.5 µl of PCR-grade water, 2.5 µl (2 mM) of dNTPs, 2.5 µl of PCR buffer, 2 µl (25 mM) of $MgCl_2$, 1.25 µl (10 µM) for each primer (AphSSUF, AphSSUR or AphLSUF, AphLSUR), 1 µl (1 U) of Taq polymerase and 2 µl of DNA template. A positive control (DNA isolate from the pure culture of the strain Ps1, genotype group B) and a negative control (blank reaction without a template) were included in each run. The PCR reaction was conducted on the Bio-Rad iCycler iQ5 thermal cycler with the following cycle settings:

3 min at 95 °C, 35 cycles of 30 s at 95 °C, 45 s at 59 °C, 30 s at 72 °C, and final step of 10 min at 72 °C. The amplification success was checked by agarose electrophoresis, and the amplified PCR products were purified by ethanol precipitation and sent for Sanger sequencing at the service laboratory. The chromatograms were inspected in Chromas v. 2.6.5 (Technelysium Pty Ltd) and the sequences were analysed with the program Mega X (Kumar et al. 2018) and compared with those of known *A. astaci* haplotypes in the NCBI GenBank database.

Table 3. Results of the microsatellite genotyping of *A. astaci*-positive DNA isolates from crayfish plague outbreaks of *A. astacus* and *A. torrentium* documented in Czechia between 2016 and 2018. For comparison of allele sizes, all reference strains from Grandjean et al. (2014) are provided. N/A: the amplification of loci was unsuccessful

Genotype (reference strain)	Host species	Locality/ Origin	SSR locus (bp)								
			Aast 2	Aast 4	Aast 6	Aast 7	Aast 9	Aast 10	Aast 12	Aast 13	Aast 14
Crayfish plague outbreaks in Czechia											
SSR-A	A. astacus	Ostrovský brook, 2016	160	103	157	207	180	142	-	194	246
SSR-A	A. astacus	Husinec dam, 2018	160	103	157	207	180	142	-	194	246
SSR-D	A. astacus	river Rožnovská Bečva 2018	N/A	131	148	203	180	142	234	N/A	250
SSR-D	A. astacus A. torrentium	Kublovský, Stroupinský, Bzovský brooks, 2018	N/A	131	148	203	180	142	234	194	250
SSR-E	A. torrentium	Radotínský brook, 2017	150	87/ 89	148/ 157	207	168/ 182	132/ 142	240	194/ 202	248
SSR-Up	A. astacus	Klenský brook, 2018	142/ 150	87	148	205/ 215	164	133/ 139	226	202	248
Reference genotypes of A. astaci											
SSR-A (VI03557)	A. astacus	Sweden, 1962	160	103	157	207	180	142	-	194	246
SSR-B (VI03555)	P. leniusculus	USA, 1970	142	87	148	215	164/ 182	132	226/ 240	202	248
SSR-C (VI03558)	P. leniusculus	Sweden, 1978	154	87	148	191	164/ 168	132	226	202	248
SSR-D (VI03556)	P. clarkii	Spain, 1992	138	131	148	203	180	142	234	194	250
SSR-E (Evira4805)	F. limosus	Czechia, 2010	150	87/ 89	148/ 157	207	168/ 182	132/ 142	240	194/ 202	248
SSR-Up	A. torrentium	Czechia, 2005	142/ 150	87	148	205/ 215	164	142	226	202	248

3. Results

Overall, six out of the ten tested mortalities were confirmed as crayfish plague outbreaks. One was recorded in 2016 in *A. astacus*, one in 2017 in *A. torrentium*, and four in 2018, in four populations of *A. astacus* and in one population of *A. torrentium* (Table 1). The case of confirmed crayfish plague episode affecting syntopic *A. astacus* and *A. torrentium* populations was considered in our study as a one outbreak consisting of three connected brooks. Each tested individual from these confirmed outbreaks was *A. astaci*-positive (agent levels A3 - A7). The pathogen wasn´t detected in any tested crayfish (four to ten individuals per site) from three mortalities of *A. astacus* and one population of *A. torrentium*; these were considered to have been caused by other reasons than crayfish plague outbreak.

The microsatellite genotyping and mtDNA sequencing of *A. astaci* strains involved in crayfish mortalities was successful for all outbreaks. Three RAPD-defined genotype groups of *A. astaci* (A, E and D) and one more distinct genotype known now yet isolated axenic cultures (Up) were identified (Table 1).

The genotype group A (with allele sizes of the microsatellite markers matching to reference strain VI03557) was identified in two crayfish plague outbreaks, both involving *A. astacus*. The first occurred in the Ostrovský brook (Central Bohemia) in May 2016, the second in the Husinec dam (South Bohemia) in July 2018. Sequences of the mtDNA markers (rnnS and rnnL) from the Husinec dam were identical to the A-haplogroup (Makkonen et al. 2018); DNA isolates from the Ostrovský brook were unavailable for mtDNA sequencing.

The genotype group E was detected in the *A. torrentium* mortality in the Radotínský brook (Central Bohemia) in September 2017. The alleles at the microsatellite markers were identical with the reference strain of *A. astaci* group E (Grandjean et al. 2014), and rnnS and rnnL sequences corresponded to the E-haplogroup (Makkonen et al. 2018).

The genotype group D was confirmed in two independent crayfish plague outbreaks from 2018. The first affected the population of *A. astacus* in the river Rožnovská Bečva (Zlín region) in May 2018. The microsatellite genotyping was successful for seven out of nine loci and matched the reference strain of the genotype group D. Amplification of two loci, Aast2 and Aast13, failed several times. The mtDNA sequencing identified the haplotype d1 of the haplogroup D. The second crayfish plague outbreak caused by the genotype group D occurred in Kublovský, Stroupinský and Bzovský brooks (Central Bohemia) in July 2018 and affected both *A. astacus* and *A. torrentium*. The allele pattern was identical to the genotype group D in eight out of nine loci; amplification was unsuccessful at the locus Aast2. The mtDNA sequence analysis revealed the presence of the haplotype d2 of the haplogroup D.

A distinct A. *astaci* strain was identified in *A. astacus* mass mortality in the Klenský brook (South Bohemia) in August 2018. The allele motif was identical in all but one microsatellite marker to the SSR-Up genotype identified in *A. torrentium* mass mortality in 2005 in the Úpořský brook, Czechia, as reported by Grandjean et al. (2014), with one exception of the locus Aast10. The isolates from the Klenský brook were consistently scored as heterozygous (with allele sizes of 133 and 139 bp), while SSR-Up was reported as homozygous by Grandjean et al. (2014) but as heterozygous by Panteleit et al. (2018). The respective rnnS and rnnL sequences matched the A-haplogroup.

In contrast, 64 tested healthy-looking individuals from five *A. torrentium* populations and 110 individuals from ten *A. astacus* populations were negative for the presence of crayfish plague pathogen (all but one with agent level A0). The exception was one *A. astacus* individual from the Balinka brook (Vysočina region, 2017), in which an agent level A1 was detected once, but not when the analysis was repeated again.

4. Discussion

Numerous crayfish mortalities occurred from 2016 to 2018 in Czechia. In our study, we documented that at least six out of these were crayfish plague outbreaks, four of which were reported in 2018 (Table 1, Fig. 1). The number of confirmed crayfish plague outbreaks in the country seems slightly higher than in the past (Kozubíková-Balcarová et al. 2014), that might be caused by higher community awareness, higher research intensity, or both (Kozubíková et al. 2008; Diéguez-Uribeondo 2009). However, the number of documented cases likely do not reflect the actual status in the country (Diéguez-Uribeondo 2009), and probably many additional crayfish plague outbreaks remain still unrecorded (Kozubíková at el. 2008). Despite of that, there might indeed be an actual increasing trend of crayfish plague outbreaks in Czechia. Such growing trend might be explained by increased spread of infected non-indigenous crayfish across the watercourses, also warmer and drier summer than in previous years might played a role (https://www.intersucho.cz/cz/; Masante et al. 2018). Especially, the drought in association with lower water levels may lead to higher organic load and oxygen deficits, which stress crayfish and increases the risk of infection. The possible contribution of stress is supported by other four documented mortalities in 2018, in which we did not detect *A. astaci* presence (Table 1). However, other crayfish pathogens may also cause mass mortalities, as recently documented on a viral disease (Grandjean et al. 2019). To identify the causes of crayfish mass mortalities, not caused by crayfish plague, further examination should be considered.

Both the analysis of nuclear microsatellite loci (Grandjean et al. 2014) and sequencing of the recently developed mtDNA markers (Makkonen et al. 2018) provided the first evidence of the presence of the *A. astaci* genotype group D in Czechia. Moreover, different haplotypes of the D-haplogroup (d1 and d2) were revealed in two distant crayfish populations in 2018 (Fig. 1). The typical vector of this

genotype group is *Procambarus clarkii* (Diéguez-Uribeondo et al. 1995; Makkonen et al. 2018), however it was also documented in *Procambarus virginalis* and *P. alleni* from aquarium pet trade (Keller et al. 2014; Mrugała et al. 2015), which probably acquired infection from *P. clarkii*. The occurrence of the *A. astaci* genotype group D in Czechia might be explained by the transmission of *A. astaci* from released infected individuals of these or other cambarid species kept in hobby aquaria or sold via ornamental trade (Mrugała et al. 2015), or from ornamental crayfish released to garden ponds (Patoka et al. 2015).

These non-indigenous species might have been escaped captivity accidentally. However after the European Union Regulation No. 1143/2014 banning of 37 Invasive Alien Species has become valid at the beginning of 2015, in some cases the regulation might have led to intentional releasing of banned pets to the wild, due to improperly awareness of hobby keepers about risks caused by non-indigenous species (Patoka et al. 2018). Potential higher number of released non-indigenous crayfish could increase the likelihood of crayfish plague transmission into open waters (Peay 2009; Chucholl 2013). This scenario is supported by two documented *P. virginalis* populations established in Czechia, although the *A. astaci* presence in these was not confirmed (Patoka et al. 2016). However, several established populations of *P. clarkii* and *P. virginalis* have already been documented in Germany (Kouba et al. 2014), and one of *A. torrentium* mass mortalities in that country was caused by the haplotype d1 of the genotype group D (Makkonen et al. 2018).

Another possible source of infection caused by this genotype group might be spread of *A. astaci* from Germany or other neighbouring country by host jumping through the country watercourses. It is more likely that the genotype group D has spread from Germany than from other neighbouring countries, however this option should also be taken into account, as these cambarid crayfish have already been established in Slovakia, Austria and Hungary (Kouba et al. 2014; Lipták et al. 2017; Gál et al. 2018).

Other RAPD-defined genotype groups from distinct Czech sites were documented too (A and E), as in the previous years. Moreover, the Up-genotype known from the 2005 outbreak in the Úpořský brook (Kozubíková-Balcarová et al. 2014) was re-identified after 13 years in a different locality (Table 3).

A. astaci from the genotype group A was detected in two mortalities of *A. astacus* in 2016 and 2018. Strains identified in this study had the same microsatellite profile as strains already documented in crayfish plague outbreaks from 2004 to 2009 (Grandjean et al. 2014). In both cases, the source of the crayfish plague pathogen remains unknown. The ability of a strain of the genotype group A to infect *P. leniusculus* was documented (Aydin et al. 2014) but its presence in any population of American crayfish was not. Nonetheless, in neither of these crayfish plague outbreaks the presence of non-indigenous crayfish species was documented. The nearest known well-established populations of American crayfish species have been recorded ca 25 km from the Husinec dam (2018 outbreak) and 72 km from

the Ostrovský brook (2016) (https://heis.vuv.cz/projekty/raci2017/). Thus, *A. astaci* does not seem to have been transmitted directly from such hosts. Even so, their presence cannot be excluded in some brooks, their small tributaries, or ponds. For some sites of older recorded crayfish plague outbreaks in the country, the presence of American crayfish has been documented much later (Kozubíková-Balcarová et al. 2014).

Another possible pathogen source for these crayfish plague outbreaks might be long-distance spread from other indigenous crayfish with chronic infection of *A. astaci* (although we did not document any such case in our study). Involvement of human activities, as transmissions of live hosts or spread of spores by, e.g., wet fishing equipment, should also be considered (reviewed in Svoboda et al. 2017). Our findings are consistent with those of Kozubíková-Balcarová et al. (2014); the RAPD-defined genotype group A, although considered less virulent than other groups (Makkonen et al. 2012, 2014), still causes crayfish mass mortalities and threatens indigenous crayfish in Czechia, as other documented genotype groups.

The RAPD-defined genotype group E was involved in *A. torrentium* outbreak in the Radotínský brook (2017) (Fig. 1), as confirmed both by microsatellites mtDNA sequences corresponding to the E-haplogroup. This RAPD-defined genotype group is linked to *Faxonius limosus* (Kozubíková et al. 2011a). Presumably, the original vector of infection in this locality might have been *F. limosus*, as their nearest well-established populations are closer than 10 km (https://heis.vuv.cz/projekty/raci2017/). This is also supported by the fact that other crayfish plague outbreaks in Czechia caused by this genotype group were documented relatively close to *F. limosus* populations (Kozubíková et al. 2011a; Kozubíková-Balcarová et al. 2014).

An unusual *A. astaci* genotype variant, labelled SSR-Up, was identified in crayfish plague outbreak affecting *A. astacus* in the Klenský brook (2018) (Table 1, Fig. 1). This variant has been documented in Czechia just once, in *A. torrentium* mass mortality in the Úpořský brook 13 years before (Grandjean et al. 2014). The allele motif identified in our study was identical to those from the Úpořský brook, with an exception of the locus Aast10 (Table 3). The locus Aast10 in the original mass mortality from the Úpořský brook was reported as a homozygote at 142 bp by Grandjean et al. (2014), whereas in our study it was constantly scored as heterozygote with allele sizes of 133 and 139 bp. Similarly, Panteleit et al. (2018) also reported heterozygosity at that locus, the only difference being slight base pair shift (135 bp and 141 bp). The observed differences in SSR-Up may be a consequence of scoring alleles in different laboratories, as in both studies the original DNA isolate from the Úpořský brook was available but results from its genotyping slightly differed (Grandjean et al. 2014; Panteleit et al. 2018). To confirm this, a further genotyping of the original material from the Úpořský brook in our laboratory is needed. The mtDNA markers from the Úpořský brook as well as from the Klenský brook matched the A-haplogroup (Makkonen et al. 2018).

As the microsatellite genotyping linked this strain as closely related to the RAPD-defined genotype group B, it was originally hypothesised that the possible source of the infection might have been *P. leniusculus* (Kozubíková-Balcarová et al. 2014; Grandjean et al. 2014). In our study, the nearest well-established *P. leniusculus* populations are 30 km away from the Klenský brook, at the borders with Austria. Thus, *P. leniusculus* cannot be excluded as a potential source of infection. Moreover, in one of them, the river Malše, heavy infection by *A. astaci* were documented (M. Mojžišová, unpublished data). However, results from genotyping of the pathogen are not available yet; thus, as a future step, genotyping of *A. astaci* from this population should be considered.

However, considering the fact, that the Úpořský brook is situated ca 170 km far from Klenský brook, and no population of *P. leniusculus* has been documented between these two locations, it seems likely that this strain was spread from other sources. Human activities might have been involved, as suggested for the Úpořský brook (Kozubíková-Balcarová et al. 2014), or the pathogen could have been transmitted from other crayfish species. The genotype Up has been identified in chronically infected *Pontastacus leptodactylus* population in the Danube Delta (Panteleit et al. 2018), thus it is not impossible that this species is the vector of this pathogen strain in Czechia. *P. leptodactylus* seems to be to some extent resistant against *A. astaci*, as supported by several studies documenting chronic infections caused by *A. astaci* genotype group A and B in this species (Maguire et al. 2016; Svoboda et al. 2014a; Kokko et al. 2018; Caprioli et al. 2018; Panteleit et al. 2018). Such chronically infected species may serve as reservoirs or vectors of crayfish plague. However, although the chronic infections have also been documented in *A. astacus* and *A. torrentium* (reviewed in Svoboda et al. 2017) and their occurrence seems relatively common for example in Croatia (Maguire et al. 2016), in our study the presence of chronic infections was observed in neither native species.

When testing for chronic infections in native crayfish species, we have examined only selected populations of *A. torrentium* and *A. astacus*. Only limited number of native crayfish individuals per populations was tested for *A. astaci* presence, and just one pereopod and one uropod were taken from each, as these species are protected. This may reduce likelihood of detecting the infection. However, the same parts and amount of the crayfish tissue from *P. leptodactylus* were enough to reveal the presence of chronic infection by *A. astaci* in Romania (Schrimpf et al. 2012), as well as to genotype the causative strain (Panteleit et al. 2018). Considering also the lack of any pathogen detection in some crayfish mortalities apparently caused by other reason than crayfish plague, it seems that chronic infection by *A. astaci* are not a frequent phenomenon in Czechia. It should be noted that in the case of one healthy-looking *A. astacus* individual from the Balinka stream, we detected the agent level A1 once (but not when the test with the same isolate was repeated); nevertheless the agent level A1 is not considered as a reliable proof of infection (Vrålstad et al. 2009). Upstream in the same river basin, however, there is an established population of *P. leniusculus*; we did not detect any trace of *A. astaci*

infection there (data of M. Mojžišová in Hladovec 2017), but low-level infections of this American species elsewhere in this region has been documented (Kozubíková et al. 2011b).

Based on performed analyses, we cannot exclude the occurrence of chronic infections by *A. astaci* in Czechia. Further examination of this phenomenon, particularly in *P. leptodactylus* populations, is demanded, due to their possible carrier status of infection. In this species, chronic infection were documented in different parts of Eastern Europe (Pârvulescu et al. 2012; Schrimpf et al. 2012; Panteleit et al. 2018), Turkey (Svoboda et al. 2012, 2014; Kokko et al. 2012, 2018), and moreover, infected *P. leptodactylus* also were documented from imports from Armenian to Czechia (Becking et al. 2015) and Italy (Caprioli et al. 2018).

In summary, in our study we documented six crayfish plague outbreaks, which probably represent just the "tip of the iceberg", and many crayfish plague outbreaks remains undetected (Kozubíková et al. 2008). We provide the first evidence of the genotype group D involved in mass mortalities of *A. astacus* and *A. torrentium* in Czechia. The mtDNA sequence analysis revealed the involvement of d1 and d2 haplotypes of D-haplogroup, which indicates two independent sources of this haplogroup in Czechia. Finding of the genotype groups A and E in *A. torrentium* and *A. astacus* was somehow expected, as these genotype groups had been documented in several crayfish mass mortalities in Czechia previously (Kozubíková-Balcarová et al. 2014). After more than a decade, the Up genotype was identified in *A. astacus* mass mortality, and we highlight the possibility that *P. leptodactylus* might be its vector. In most of these documented crayfish plague outbreaks, except the one caused by the genotype E of *A. astaci*, the original source of infection remains unclear, as no non-indigenous crayfish were observed in immediate vicinity of crayfish mass mortalities. Further, our study for the first time inspected the possible presence of chronic infections by *A. astaci* in native crayfish in Czechia. Although this phenomenon was not observed, its potential occurrence cannot be excluded and further examination of the additional candidate species, *P. leptodactylus,* should be performed.

Our findings confirm that the number of documented crayfish plague outbreaks in Czechia is increasing rather than decreasing, and apparently is at present a greater problem in conservation of native crayfish than for example water pollution. Screening for *A. astaci* and non-indigenous crayfish should become a part of conservation strategy for indigenous crayfish and the application of eDNA approach (Wittwer et al. 2018; Strand et al. 2019), should be considered, as it seems highly effective for this purpose.

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Príloha 1

Lipták B., **Mojžišová M.,** Gruľa D., Christophoryová J., Jablonski D., Bláha M., Petrusek A., Kouba A. (2017): Slovak section of the Danube has its well-established breeding ground of marbled crayfish *Procambarus fallax* f. *virginalis*. *Knowledge and Management of Aquatic Ecosystems*, 418: art. no. 40.

Autorsky podiel: V tejto štúdii som testovala vzorky rakov *Procambarus virginalis* a *Faxonius limosus* na prítomnosť patogénu račieho moru.

Príloha 2

Putra M.D., Bláha M., Wardiatno Y., Krisanti M., Yonvitner, Jerikho R., Kamal M.M., **Mojžišová M.,** Bystřický P.K., 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: Marine and Freshwater Ecosystems*, 28: 1434-1440.

Autorsky podiel: V tejto štúdii som testovala vzorky rakov *Procambarus clarkii*, tak ako aj vzorky krabov *Parathelphusa convexa* a kreviet *Macrobrachium lanchesteri* na prítomnosť patogénu račieho moru. Testovanie prebiehalo v spolupráci so študentom P. K. Bystřický, ktorého som v rámci testovania učila metódy detekcie.