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Research Article

Distribution of *Aphanomyces astaci* Schikora, 1906, the causative agent of crayfish plague, in the Plitvice Lakes National Park, Croatia

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Abstract

Numerous native European freshwater crayfish populations are in decline due to the lethal disease crayfish plague caused by the pathogen *Aphanomyces astaci* (Oomycetes). Presence of this pathogen has already been recorded in the Plitvice Lakes National Park (NP), Croatia, where two protected native crayfish species, the noble crayfish *Astacus astacus* and the stone crayfish *Austropotamobius torrentium* occur. Data presented in this manuscript are part of a two-year monitoring project of the pathogen in the NP. Previously, we have reported the overall prevalence of *A. astaci* positive individuals in *A. astacus* and *A. torrentium* populations (14% and 2% of tested crayfish, respectively) within the NP. Here, we report the detailed distribution of the pathogen within the NP and differences in its prevalence in populations of both crayfish species collected at different locations within the NP. Moreover, we have identified the *A. astaci* genotype present within the NP and discuss our findings in the context of crayfish plague related management activities in the NP. The majority of pathogen records were from the *A. astacus* populations which were all in close proximity, and this presumably facilitated the pathogen spread among them. Prevalence of *A. astaci* positive *A. astacus* varied in different populations from 10% to 18%. In comparison, only one out of three tested *A. torrentium* populations, in the proximity of the *A. astacus* populations, was *A. astaci* positive (prevalence 17%), while in the other two geographically distant populations pathogen was not recorded. The microsatellite genotyping identified the presence of *A. astaci* haplogroup A (As genotype). Although known for its low virulence, *A. astaci* of haplogroup A could still cause mass mortalities, especially if combined with other stressors that can impair crayfish health. Obtained results were used as a baseline for the development of monitoring protocol for *A. astaci* and were incorporated in the management plan for protection of vulnerable native species in the NP. We present the proposed management activities for prevention of unintentional spread of the crayfish plague pathogen to adjacent streams that are inhabited by pathogen free native crayfish populations.

Key words: indigenous crayfish species, *Astacus astacus*, *Austropotamobius torrentium*, non-destructive *Aphanomyces astaci* detection, invasive alien species, crayfish monitoring

Introduction

European indigenous crayfish species (ICS) suffer substantial population declines throughout Europe (Kouba et al. 2014) and are threatened by

different factors such as habitat loss, climate change, overfishing, non-indigenous crayfish species (NICS) and crayfish plague epidemics (Alderman 1996; Holdich et al. 2009). Crayfish plague is a disease caused by the pathogenic oomycete *Aphanomyces astaci* Schikora, 1906, which is considered one of the world's worst invasive alien species due to its fast spread and destructive impact (Lowe et al. 2000). This pathogen is frequently transmitted by NICS that normally act as its permanent carriers and are known often to be more resistant to infection than ICS (Aydin et al. 2014; Sandström et al. 2014). ICS most often develop the disease through contact with crayfish plague carriers and *A. astaci* zoospores (Unestam and Weiss 1970; Makkonen et al. 2014; Becking et al. 2015). To date, five different genetic groups (namely A, B, C, D and E) have been identified by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) (Huang et al. 1994; Diéguez-Uribeondo et al. 1995; Kozubíková et al. 2011; Viljamaa-Dirks et al. 2013), and confirmed by microsatellite genotyping (Grandjean et al. 2014) and mtDNA ribosomal subunit differences (Makkonen et al. 2018). The different *A. astaci* haplogroups differ in their original hosts and virulence severity. *Aphanomyces astaci* of haplogroup A, as defined by Makkonen et al. (2018), is considered the first *A. astaci* haplogroup to have invaded Europe and it also includes PsII genotype. It was first isolated from infected crayfish of the genus *Astacus*, however its original host is still unknown (Huang et al. 1994). The rest of the haplogroups are linked to NICS. Haplogroup B originates from the signal crayfish *Pacifastacus leniusculus* (Dana, 1852) (Huang et al. 1994). Haplogroup D, which consists of two haplotypes d1 and d2, originates from the red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Diéguez-Uribeondo et al. 1995; Rezinciuc et al. 2014). Haplogroup E originates from the spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817) (Kozubíková et al. 2011).

Strains of different haplogroups may vary in virulence, with *A. astaci* of haplogroup B being generally more virulent compared to *A. astaci* of haplogroup A as shown in studies on *Astacus astacus* (Linnaeus, 1758) (Makkonen et al. 2014; Becking et al. 2015; Svoboda et al. 2017). Some ICS populations show resistance towards some strains of *A. astaci* (mostly haplogroup A; Viljamaa-Dirks et al. 2011; Svoboda et al. 2012; Makkonen et al. 2014). Latent *A. astaci* infection with haplogroup A has been detected in some *Pontastacus leptodactylus* (Eschscholtz, 1823) populations in Turkey and Romania (Kokko et al. 2012, 2018; Pârvulescu et al. 2012), as well as in populations of *A. astacus* in Finland (Jussila et al. 2020) and Croatia (Maguire et al. 2016; Pavić et al. 2020), *Austropotamobius torrentium* (Schrank, 1803) in Slovenia (Kušar et al. 2013; Jussila et al. 2017) and Croatia (Maguire et al. 2016; Pavić et al. 2020), and *Austropotamobius pallipes* (Lereboullet, 1858) in Italy (Manfrin and Pretto 2014) and Croatia (Maguire et al. 2016). The resistance observed in these populations has frequently been attributed to the adaptation of crayfish immune system to

the haplogroup A *A. astaci* due to its long presence in European freshwater habitats (Jussila et al. 2011; Makkonen et al. 2012; Jussila et al. 2015). Other than that, chronic infections in ICS populations may develop as a consequence of *A. astaci* decreased virulence since its first invasion of European freshwaters (Makkonen et al. 2012). Moreover, resistance of native European crayfish to some of the more virulent strains has also been reported, i.e., *A. pallipes* to haplogroup D (Martín-Torrijos et al. 2017) or *P. leptodactylus* to haplogroup B (Jussila et al. 2020; Ungureanu et al. 2020). During the latent crayfish plague infection, mass mortalities or gross signs (e.g. daytime activity, limb loss, or abdominal paralysis (Alderman et al. 1987)) do not occur, while gross signs of disease (i.e. melanisation spots) can be observed on the crayfish carapace (Martín-Torrijos et al. 2017; OIE 2019). However, factors such as moulting, reproduction, altered environmental conditions and attack by different parasites can decrease crayfish immune defence and lead to acute crayfish plague development, even in more resistant NICS species (Thörnqvist and Söderhäll 1993; Cerenius et al. 2003; Aydin et al. 2014; Edsman et al. 2015; Thomas et al. 2020).

Spread of *A. astaci* among protected and endangered ICS is currently one of the major challenges in European crayfish conservation. Native European crayfish are highly susceptible to anthropogenic pressure onto their habitats as well as to spread of NICS and the pathogens they carry, such as *A. astaci*, and maintaining their current biodiversity requires development of effective management programs (Souty-Grosset et al. 2004; Kozák et al. 2011). Control of NICS and control of pathogen spread is among most important tasks within such management programs because introduction of pathogen into ICS population can decimate it in a very short time period. Up to date different approaches with different successes, aiming to control NICS and crayfish plague spread have been proposed and tested, e.g. biological control (Aquiloni et al. 2010; Cecchinelli et al. 2012), trapping (Green et al. 2018), electric shock treatment (Peay et al. 2014), biocide treatments (Peay et al. 2019) and physical barriers (Krieg and Zenker 2020; Krieg et al. 2020). None of these approaches ensure complete success in controlling NICS, but education and continuous monitoring are recognised as inseparable part of effective programs (Souty-Grosset et al. 2004; Nightingale et al. 2017; Krieg and Zenker 2020).

Development of effective management programs is especially important for populations in protected areas, such as national parks, since they are often regarded as potential refugia sites that could secure the long-term survival of native crayfish species and their diversity (Nightingale et al. 2017; Ferrante et al. 2018). Therefore, conservation programs in protected areas require combination of different activities (Mozsár et al. 2021), from direct management activities such as monitoring of pathogen presence and the spread of invasive crayfish, to education of employees regarding their practices related to unintentional spread of invasive species and pathogens. To

avoid unnecessary sacrificing of endangered ICS, a new trend in the *A. astaci* monitoring is application of non-destructive approaches, i.e. detection of the pathogen from sampled pereopods and uropodes (Schrimpf et al. 2012), in the biofilm present on the cuticle surface (Manfrin and Pretto 2014; Pavić et al. 2020), or in the eDNA isolated from the water (Strand et al. 2019; Rusch et al. 2020).

In this study we present the results of non-destructive *A. astaci* monitoring and subsequent development of activities for NICS and pathogen control in order to preserve ICS in protected area – National Park Plitvice Lakes (hereafter NP), one of the most well-known Croatian National parks. We have previously demonstrated the *A. astaci* presence in the Plitvice Lakes NP where two nationally and internationally protected (OG 80/13 and OG 88/14; Appendix III of the Bern Convention, Appendices II and V of the Habitat Directive (92/43/EEC and 97/62/EU)) ICS occur: the noble crayfish, *Astacus astacus* and the stone crayfish, *Austropotamobius torrentium* (Maguire and Gottstein-Matočec 2004; Maguire et al. 2013). The crayfish plague has been detected in the *A. astacus* populations within the NP (Maguire et al. 2016). However, this was based on a small sample size and traditional (i.e., destructive) *A. astaci* detection method (Oidtmann et al. 2006; Maguire et al. 2016). In 2017 and 2018, the presence of the pathogen was confirmed in the NP, this time on both ICS and in multiple locations (Pavić et al. 2020). Here we applied the recently developed non-destructive method based on analysis of cuticle swabs that enabled sampling of large number of crayfish individuals (Pavić et al. 2020) and crayfish plague monitoring without the need to kill crayfish and obtain cuticle sample. Results presented in Pavić et al. (2020) showed that overall 14% of tested *A. astacus* and 2% of tested *A. torrentium* were *A. astaci* positive.

While the manuscript by Pavić et al. (2020) was focused on the applicability of the recently developed method in *A. astaci* detection during monitoring of wild crayfish populations, the present study aims to analyse in detail the prevalence and distribution of *A. astaci* in *A. astacus* and *A. torrentium* populations in multiple waterbodies within the NP. Additional aim of the current study was to identify *A. astaci* genotype. Furthermore, based on the results obtained here and in the study by Pavić et al. (2020), we propose potential activities, applicable to any region, for the crayfish plague spread limitation and NICS management plan that will aid in the conservation of *A. astacus* and *A. torrentium* within the NP.

Materials and methods

Study area

The Plitvice Lakes National Park, founded in 1949, is the oldest and the largest national park, situated in the Dinaric karst region of continental Croatia. Within the NP there are numerous springs, streams and 16 lakes

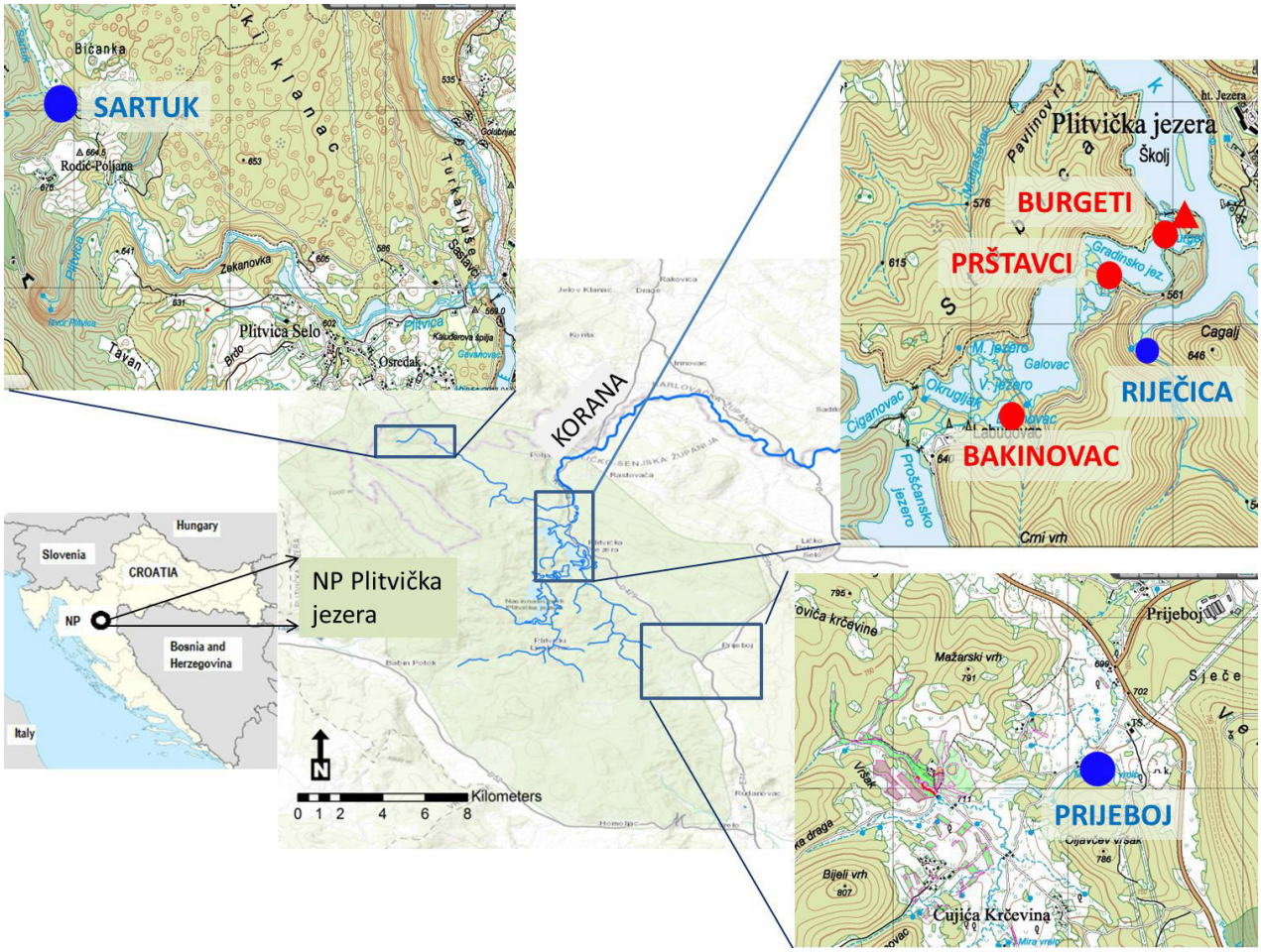

Figure 1. Position of studied sites within Plitvice Lakes National Park. Blue dots – sampled *Austropotamobius torrentium* populations; red dots – sampled *Astacus astacus* populations in 2017 and 2018; red triangle – *A. astacus* population sampled in 2012 and 2013. For coordinate details see Supplementary material Table S2.

divided into the Upper and Lower lakes (Figure 1). They are separated by tufa barriers and waterfalls – fundamental natural phenomenon of the NP. Due to their uniqueness and outstanding universal value, the NP was listed in 1979 as UNESCO Word Heritage Site (Roglić 1974; Biondić et al. 2010).

Sampling

Crayfish were trapped once a month (from March to October, during the period of crayfish activity) with baited LiNi and hand-made traps (Maguire et al. 2002) that were left in the water overnight. At each site GPS coordinates were recorded for geographical presentation of populations' distribution that was produced in ArcGIS 10.3 program package. Sampling was conducted at six different locations within the NP where crayfish presence was previously detected (Maguire et al. 2013): three locations for *A. astacus* and three locations for *A. torrentium* (Figure 1, Table 1). Samples of cuticle-associated epibiotic communities from each crayfish were collected by brushing their surface with a sterile toothbrush as described in Pavić et al. (2020). After brushing, the crayfish were returned to their habitat, except those with potential signs of latent *A. astaci* infection

Table 1. Details about crayfish species, sampling localities, number of tested crayfish per species (including number of tested cuticles and swabs), with numbers of *A. astaci* positive in total number of tested crayfish per location (including details on positive sample origin; cuticle or swab) and 95% confidence level range for background population infection level (%) based on binomial probability estimation.

Crayfish species	Locality	Total (cuticle + swab)	No. infected (cuticle + swab) / Total tested	95% confidence level range
Astacus astacus	Bakinovac (Veliko jezero)	114 (17 + 97)	0/4	0–60
	Burgeti		9 (6 + 3)/93	5–18
	Prštavci		3 (2 + 1)/17	4–43
Austropotamobius torrentium	Prijeboj	46 (1 + 45)	0/22	0–15
	Riječica		1 (1 + 0)/6	1–64
	Sartuk		0/18	0–19

(e.g., abundant melanisation spots on leg joints and abdomen). These potentially infected crayfish were killed (rapid cutting through their nervous system) and pieces of their cuticle were taken for further analyses (Oidtmann et al. 2006). In total, 160 crayfish individuals were tested, out of which 114 were *A. astacus* and 46 were *A. torrentium*, with a total of 18 cuticle and 142 swab samples (Table 1).

DNA extraction and A. astaci *detection*

DNA was extracted from swabs and cuticle by using NucleoSpin® Microbial DNA kit (Macherey Nagel, Germany), as described in Pavić et al. (2020). *Aphanomyces astaci* DNA was detected in isolated material by PCR with primers 42 and 640 following protocol by Oidtmann et al. (2006), except the annealing temperature was increased from 59 °C to 61 °C to increase specificity as described in Pavić et al. (2020). We have used a high-yield DNA polymerase (EmeraldAmp MAX HS PCR Master Mix, Takara), enabling a highly sensitive *A. astaci* detection by standard PCR. Relative quantification of *A. astaci* DNA in PCR-positive samples was done by qPCR with primers AphAstITS-39F, AphAstITS-97R and MGB probe AphAstITS-60T (Vrålstad et al. 2009). Based on the PCR forming units (PFU) values, qPCR results were classified into semi-quantitative categories of pathogen load, ranging from A0 (no traces of *A. astaci* DNA) to A7 (high amount of *A. astaci* DNA in the sample) (Vrålstad et al. 2009). Level A1 was regarded as *A. astaci* negative, since such a low PFU value (corresponding to 5 PFU) may indicate a very low pathogen load but also a false positive or a minor contamination (Filipová et al. 2013). Only samples positive by standard PCR were tested by qPCR since previous studies (Tuffs and Oidtmann 2011) showed that the difference in sensitivity between qPCR and standard PCR is one order of magnitude. The lowest agent level recorded in our study by qPCR was A2, meaning that we were able to detect all positive samples (agent level A2 and higher, Vrålstad et al. 2009) already by standard PCR.

Microsatellite genotyping

For further genotype characterisation, the microsatellite loci (Aast 2, Aast 4, Aast 6, Aast 7, Aast 9, Aast 10, Aast 13 and Aast 14) were used and analyses

were done following procedures described in Grandjean et al. (2014). Genotyping included only those samples that had moderate to high *A. astaci* agent level (≥ A3) (Vrålstad et al. 2009), as previously determined by qPCR.

Binomial probability estimation

In order to estimate the probable infection rate among the background population, we performed binomial probability estimation that predicts 95% confidence levels for the infection rate (Zar 1996). The analysis was performed in a program AV Bio-Statistics 4.9 Professional.

Results

The obtained results established the presence of the pathogen *A. astaci* in three out of six sampled locations in the NP, two inhabited by *A. astacus* (Burgeti and Prštavci) and one inhabited by *A. torrentium* (Riječica). In all tested *A. astacus* individuals from Bakinovac (Veliko jezero) (n = 4), and *A. torrentium* individuals from Prijeboj (n = 22) and Sartuk (n = 18) pathogen was not detected. The binomial probability calculation estimated that between 5% and 18% of the *A. astacus* population from Burgeti site (93 crayfish tested) might be infected (Table 1). While for other two sites with smaller *A. astacus* sample size (Bakinovac – 4 crayfish, Prštavci – 17 crayfish) results of binomial probability calculation estimates indicated a possibility of rather high potential infection percentage among the background population (up to 60% in Bakinovac, and 43% in Prštavci). Similarly, high potential infection rate was established for *A. torrentium* populations from Riječica (up to 64%, 6 crayfish tested); while for populations from Prijeboj and Sartuk smaller estimated background infection levels were calculated (up to 15% and 19%, respectively) (Table 1).

Results of the standard PCR were confirmed by qPCR. Agent level of the *A. astaci*-positive samples varied from A2 to A5. Microsatellite genotyping was performed for the samples with agent level ≥ A3, and for these samples loci sizes were identical to a typical *A. astaci* of haplogroup A (As genotype) (Supplementary material Table S1). The loci sizes were as follows: Aast2 = 160; Aast4 = 103; Aast6 = 157; Aast7 = 207; Aast9 = 180; Aast10 = 142; Aast13 = 194; Aast14 = 246.

Discussion

Our two-year survey study, carried out in 2017 and 2018, confirmed the presence of *A. astaci* in the NP. Obtained results were partly presented in Pavić et al. (2020), where applicability of a non-destructive method for *A. astaci* detection in the field was tested on a large number of crayfish. In the present study we provide detailed report on the distribution and prevalence of the pathogen in different localities within the NP, results on genotyping of positive (agent level ≥ A3) samples, and based on obtained

results, provide a proposal of activities for *A. astaci* monitoring which were incorporated into management plan of the NP.

Presence of *A. astaci* in the NP was recorded for the first time in 2012, during the survey of pathogen's distribution in Croatian ICS and NICS populations (Maguire et al. 2016). This study included a small number of *A. astacus* individuals (14) from a single location in the NP (Burgeti site, Figure 1), and established that 71% of tested *A. astacus* (10 out of 14) were infected by *A. astaci* of haplogroup A. Even though there are no exact literature data on the presence of the *A. astaci* in the NP before 2012, historical data reveal that two crayfish plague outbreaks hit ICS populations in the continental part of Croatia in 1880s and 1960s (Anonymous 1901; Plančić 1973). Since there were no records of NICS until early 2000s in Croatia, and viable ICS populations existed (Maguire and Gottstein-Matočec 2004) we assume that previous outbreaks were caused by *A. astaci* of haplogroup A, that is generally less virulent compared to other haplogroups connected to NICS (Makkonen et al. 2014; Becking et al. 2015; Svoboda et al. 2017). This would also imply that pathogen *A. astaci* of haplogroup A has been present in the NP for many decades.

In comparison to the earlier study (Maguire et al. 2016), data from the present monitoring survey (2017/2018) were obtained from a much larger sample of *A. astacus* (114 individuals) from 3 localities as well as of *A. torrentium* (46 individuals) from 3 localities, and we confirmed the presence of haplogroup A in the NP. In the case of *A. astacus*, only 11% of all tested *A. astacus* were *A. astaci* positive, while at the Burgeti site (analysed both in Maguire et al. (2016) and in this study), the presence of the pathogen was confirmed for 10% of tested individuals, and the binomial probability calculation estimated that up to 18% of the population might be infected. Discrepancy in the percentage of infected *A. astacus* individuals in the present (10%) and previous (71%; Maguire et al. 2016) research is probably a result of different sampling approaches (non-destructive vs. destructive method), since in both studies qPCR was used for pathogen detection and microsatellite markers for genotyping. In 2012 we have performed the sampling by killing crayfish in order to take a piece of cuticle for *A. astaci* detection (Oidtmann et al. 2006). Therefore, we sampled only those crayfish that exhibited visible symptoms of latent *A. astaci* infection (e.g., melanisation, day activity), and targeted a small sample size of suspected individuals within a population to avoid sacrificing protected species within protected area. In the present research we applied non-destructive method (swab samples) and crayfish were monitored for *A. astaci* presence regardless of the existence of the gross signs of infection and the large numbers of crayfish were tested (114 *A. astacus* and 46 *A. torrentium*) which enabled a more complete insight into infection status of populations. Thus, the newly developed non-destructive method (Pavić et al. 2020) was demonstrated to be beneficial since sampling bias towards

individuals with crayfish plague gross signs, inevitable when using traditional destructive sampling, could be intentionally avoided. This adds to a new trend of development of less destructive approaches of *A. astaci* detection in large number of ICS individuals, such as previously proposed cutting of uropodes and melanised pereopods instead of sacrificing whole animals (Schrimpf et al. 2012).

During the present study, a new site (Prštavci, Figure 1) with chronic *A. astaci* infection of *A. astacus* population was detected. This site is situated 400 m upstream from the infected Burgeti site, on the southern part of the same lake, indicating that crayfish from both localities are possibly in contact (Figure 1) and consequently share the pathogen. On the other hand, *A. astaci* was not detected on the Bakinovac site (Table 1). The Bakinovac site is 900 m further upstream from the Prštavci site (and 1300 m away from Burgeti) and is situated at another lake (Figure 1) that is separated from the Burgeti and Prštavci sites by cascade of waterfalls that could serve as a possible barrier for upstream dispersal of infected crayfish (Figure 1). However, it has to be noted that we have trapped a small sample (n = 4) at Bakinovac (Table 1), which is not large enough for a reliable confirmation of the infection free status of the population, as shown by the binomial probability estimates, which had among the widest range of potential background infection, with a maximum up to 60%.

Pathogen was also detected in the *A. torrentium* population in the Riječica Stream with incidence of 17% (1 infected individual out of 6 tested). The crayfish sample size in this site was small and additional sampling would allow more confident assessment of the population infection status, since the results of a binomial probability analysis indicated a wide range of possible background population infection (1–64%). This is understandable since estimates depend upon the sample size – the smaller the sample size the wider the probability range. Due to low infection level (Table S1) genotyping could not be performed, however we could assume that crayfish in this population also carry *A. astaci* of haplogroup A that is known for its lower virulence (Makkonen et al. 2014; Becking et al. 2015; Svoboda et al. 2017) and enable population's viability despite the recorded *A. astaci* presence (Kušar et al. 2013). Also, the Riječica Stream flows into the same lake where pathogen was recorded in the *A. astacus* populations (Prštavci and Burgeti sites) (Figure 1), so it is possible that the *A. torrentium* population from Riječica Stream came into contact with *A. astacus* from the Burgeti or Prštavci sites which enabled transfer of *A. astaci* zoospores into population (Alderman and Polglase 1985; Svoboda et al. 2017). Another possibility is that the pathogen was unintentionally introduced into the stream through infected fishing equipment (Alderman et al. 1990; Diéguez-Uribeondo et al. 2006; Kozubíková et al. 2009; Pârvulescu et al. 2012).

At the other two *A. torrentium* sites, Prijeboj and Sartuk, that are geographically distant from infected sites (Figure 1), pathogen was not

detected. These two populations present two deeply divergent molecular phylogroups within *A. torrentium* possessing high genetic diversity that require special attention and strict protection (Klobučar et al. 2013; Lovrenčić et al. 2020) and should be prioritised in the future crayfish plague monitoring, with the emphasis on prevention of pathogen transmission into those populations.

Thus both *A. astacus* and *A. torrentium* populations in the NP demonstrate latent infection which could potentially under stressful conditions (e.g., during moulting), develop and cause acute episodes of mass mortality as recorded in numerous European countries (Caprioli et al. 2018; Mojžišová et al. 2020). Therefore regular monitoring of crayfish plague within the populations of crayfish in the NP is of paramount importance.

In addition to existing latent infection within the NP, *P. leniusculus* represents potential threat to native crayfish in the NP. *Pacifastacus leniusculus* was illegally introduced into the lower reaches of the Korana River, a karstic river that springs from the last lake in the NP (Figure 1) (Hudina et al. 2017) and is spreading upstream through the river towards the NP (Dragičević et al. 2020). This species is known to be a carrier of the highly virulent *A. astaci* of haplogroup B (Huang et al. 1994) that causes fast development of the disease and death of native crayfish species (Makkonen et al. 2012; Jussila et al. 2015). *Aphanomyces astaci* of haplogroup B was involved in four out of five mass mortalities of the *A. pallipes* characterised in France in 2014–2015 (Grandjean et al. 2017), as well as in numerous other crayfish plague outbreaks across Europe (Vrålstad et al. 2014; Martín-Torrijos et al. 2019; Strand et al. 2019), and therefore represents a prominent danger for native European crayfish species. Thus, in addition to crayfish plague monitoring within the NP, occasional genotyping of the *A. astaci* haplogroup, as well as monitoring of upstream dispersal of the signal crayfish is required.

Finally, based on our results some activities that we proposed were incorporated into the management plan of the NP (Management plan for NP Plitvička jezera, 2019–2028; hereon NP Management Plan). Good collaboration between scientific community and the managers of protected areas is of the utmost importance for successful activities planning (Souty-Grosset et al. 2004). A series of activities were developed and proposed to prevent the spread of *P. leniusculus* and *A. astaci* of haplogroup B into the NP, as well as to prevent further spread of *A. astaci* of haplogroup A within the NP. They include regular monitoring of *A. astaci* in the endangered and protected ICS populations in the NP (and its surroundings) using a non-destructive *A. astaci* detection method (Pavić et al. 2020). The long-term monitoring programs are recognised as an important element in management plans (Nightingale et al. 2017). Also, the disinfection of fishing equipment and other gear (e.g., traps, boots, nets) used by scientists

and the NP staff, whose activities are connected to waterbodies in the NP, has been marked as compulsory within the NP Management Plan to avoid unintentional introduction and spread of *A. astaci* (Jussila et al. 2014). Furthermore, the continuous monitoring of signal crayfish dispersal at the upstream invasion front in the Korana River, ideally coupled with potential control measures such as intensive trapping and removal, or sterile male release technique (Johović et al. 2020) was prescribed. Additionally, relocation of the native crayfish within the NP into ark sites, that should be carefully chosen (Peay 2009), could enable their long term survival as proposed in Nightingale et al. (2017). Finally, education of NP employees, local inhabitants, and visitors is exceedingly important in order to avoid introduction of invasive crayfish species into waterbodies of NP (Nightingale et al. 2017). For this purpose we have created educational leaflets on crayfish plague for tourists (Supplementary material Appendix 1), that were distributed to visitors of the NP with the ticket purchase. Leaflets can also be used by the employees of NP and other nature protection institutions, as well as scientists, in order to facilitate the education on crayfish plague and NICS.

In conclusion, the present study revealed detailed distribution of *A. astaci* and its prevalence in populations of two ICS present in the NP. Also, obtained results were used for development of management activities dedicated to *A. astaci* monitoring and prevention of disease spread. Our study demonstrated the applicability of innovative methods based upon scientific research in conservation programs for native endangered crayfish.

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Authors' Contribution:

I.M., A.B., S.H. – research conceptualization; I.M., A.B., S.H. – sample design and methodology; I.M., A.B., S.H., D.P., J.J., I.Š. – investigation and data collection; D.P., F.G., J.J., I.M., A.B., S.H. – data analysis and interpretation; D.P., I.M., A.B., S.H. – original draft; All authors – writing, review and editing.

Ethics and Permits

The research pertaining to this article was conducted with the permission from the Ministry of Environment and Nature Protection of the Republic of Croatia (UP/I-612-07/16-48/151) and the Plitvice Lakes National Park (10308).

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Supplementary material

The following supplementary material is available for this article:
Table S1. Details of *Aphanomyces astaci*-positive crayfish.
Table S2. Geographic coordinates of studied sites.
Appendix 1. Leaflet.

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