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Prevalence of the pathogen *Aphanomyces astaci* in freshwater crayfish populations in Croatia

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ABSTRACT: The Oomycete *Aphanomyces astaci* is an obligate crayfish parasite that co-evolved with American crayfish species, and they therefore generally live in a balanced relationship. On the contrary, European native crayfish are highly susceptible to *A. astaci*, and infestation with it causes development of the lethal disease termed crayfish plague. Until now, 5 *A. astaci* strains have been described from the freshwater crayfish present in Europe. In this study we aimed to investigate the occurrence of the pathogen *A. astaci* in Croatian native and non-native crayfish populations, as well as to genotype established strains using microsatellite markers and obtain information on the pathogen's epidemiology. Our results showed that the pathogen is widespread in both native and non-native crayfish populations. Agent level, when positive, in non-native crayfish was generally low; in native species it was higher. Genotyping from microsatellites proved the presence of the B (Ps) strain in non-native species (*Pacifastacus leniusculus*), while the A (As) strain was detected from viable native species (*Astacus astacus* and *Austropotamobius torrentium*) that are distributed in areas lacking non-native crayfish. The genotype from *A. torrentium* differed from a typical A (As) by 1 allele. Strain B (Ps) was identified in native *Astacus leptodactylus* from the population that co-occurs with *P. leniuscuslus*. Interestingly, in 1 *A. leptodactylus* population both A (As) and B (Ps) strains were present.

KEY WORDS: Crayfish plague · Non-native crayfish · Native crayfish · Genotyping

INTRODUCTION

Freshwater crayfish are the largest invertebrates in freshwater habitats. They are considered keystone species, as their size and role in freshwater systems make them highly important for ecosystem stability (Reynolds 2006, Reynolds & Souty-Grosset 2012). European indigenous freshwater crayfish (ICS) include 2 genera and 5 species (*Astacus astacus*, *Astacus leptodactylus*, *Astacus pachypus*, *Austropotamobius pallipes* and *Austropotamobius torrentium*). Once widely distributed and abundant, the ICS are facing significant declines in their area of occurrence and numbers throughout Europe (Holdich et al. 2009). These declines are severe to the extent that 3 out of 5 species are listed in the Bern convention, EU Habitat directive and IUCN Red List of Threatened Species (Edsman et al. 2010, Füreder et al. 2010).

European ICS are mainly threatened by habitat deterioration, water quality decline, climate changes, the presence of non-indigenous American crayfish species (NICS) and the crayfish plague (Holdich et al. 2009). Apart from being more aggressive, fertile and fast growing, American NICS are also spreading vectors of the pathogen *Aphanomyces astaci* (Oomycetes), the causative agent of a disease called crayfish plague, which can be transmitted not only via direct

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contact with ICS, but also through waterways, fishing equipment and fishes (Oidtmann et al. 2002, Pârvulescu et al. 2012).

A. astaci is an obligate crayfish parasite that co-evolved with American freshwater crayfish, and therefore, in normal living conditions, they live in a balanced relationship (Unestam 1969, Söderhäll & Cerenius 1999, Diéguez-Uribeondo et al. 2006). On the contrary, the pathogen is lethal for European ICS because their immune response is not efficient enough to stop infection and successfully defend against the disease (Cerenius et al. 2003). Consequently, mass mortalities and dramatic declines have occurred in European ICS populations (Souty-Grosset et al. 2006).

To date, 5 distinct genotype groups of *A. astaci* have been distinguished using random amplified polymorphic DNA (RAPD) analysis (Huang et al. 1994, Diéguez-Uribeondo et al. 1995, Kozubíková et al. 2011a). Group A (also referred to as the As or 'old' type) comprises *A. astaci* strains isolated from infected European crayfish species (*A. astacus* and *A. leptodactylus*). It is commonly assumed that the Group A genotype originates from the first introduction of crayfish plague to Europe (Huang et al. 1994). Its original American host still remains unknown. Groups B and C (also referred to as the PsI and PsII genotypes, respectively) comprise strains originating from *Pacifastacus leniusculus* found in California, USA, and Canada, respectively (Huang et al. 1994). Group D (referred to as the Pc genotype), clearly showing an adaptation to warmer waters, has been isolated from *Procambarus clarkii* (Diéguez-Uribeondo et al. 1995). Group E represents strains isolated from *Orconectes limosus* (Kozubíková et al. 2011a), and it is referred to as the Or genotype. Other dominant molecular markers such as amplified fragment-length polymorphisms (AFLPs) have also confirmed the genetic distinctions between these groups (Rezinciuc et al. 2013). Recently, Grandjean et al. (2014) developed a set of 10 microsatellites allowing characterisation of strains directly from DNA extracted from a piece of infected cuticle. These molecular markers provide the opportunity to improve our knowledge of the pathogen's epidemiology by the analysis of ancient samples conserved in alcohol (Vrålstad et al. 2014) or to characterise strains involved in more recent outbreaks (Grandjean et al. 2014).

Parallel to the improvement of methodology for crayfish plague detection, several studies have been performed on the occurrence of *A. astaci* in Europe. Investigations were mainly conducted on NICS populations as potential carriers of the disease (Kozubíková et al. 2011b, Vrålstad et al. 2011, Pârvulescu et al. 2012, Schrimpf et al. 2013a, Keller et al. 2014) but also included a few ICS populations (Pârvulescu et al. 2012, Kušar et al. 2013, Gruber et al. 2014, Kozubíková-Balcarová et al. 2014). In a study that included both ICS and NICS (*Austropotamobius pallipes*, *Pacifastacus leniusculus*, *Orconectes limosus*, *O. immunis* and *Procambarus clarkii*) and was the most comprehensive, considering the number of crayfish and populations analysed, Filipová et al. (2013) reported that >50% of *P. leniusculus* populations harbour infected individuals. Also, Schrimpf et al. (2013b) reported that co-existence of ICS and NICS in mixed populations is possible if NICS are pathogen free. Further, some recent studies discovered viable ICS populations latently infected with *A. astaci* (Jussila et al. 2011, Kokko et al. 2012, Svoboda et al. 2012, Kušar et al. 2013, Makkonen et al. 2014).

In Croatia, the crayfish plague has been sporadically mentioned in the literature as a possible cause of crayfish mass mortalities, but so far no scientific approach has been applied (Anonymous 1897, 1899, Plančić 1973), and no data on prevalence of *A. astaci* in crayfish populations are available. Croatian freshwaters are inhabited by 4 ICS (*A. astacus*, *A. leptodactylus*, *A. pallipes* and *A. torrentium*) and 3 NICS (*O. limosus*, *P. leniusculus* and *P. fallax* f. *virginalis*) (Maguire et al. 2011, Samardžić et al. 2014). Moreover, mixed populations of NICS and ICS have also been recorded in some water bodies (Hudina et al. 2009, 2011, 2013).

Therefore, the aim of this study was to (1) confirm that the crayfish plague is/was present and involved in disease outbreaks and (2) gain insight into the prevalence of the pathogen *A. astaci* in randomly chosen populations of both ICS and NICS inhabiting Croatian freshwaters, including supplementary samples of the Australian species *Cherax quadricarinatus* obtained from the only known European wild population existing in Slovenia (Jaklič & Vrezec 2011). Additionally, genotyping of strains has been performed by the use of microsatellite markers to obtain more detailed information on pathogen epidemiology.

MATERIALS AND METHODS

Crayfish were collected by traps or by hand, randomly during field work on different water bodies belonging to both the Black Sea and the Adriatic Sea drainages between 2003 and 2013 (Fig. 1). Samples included individuals appearing healthy as well as

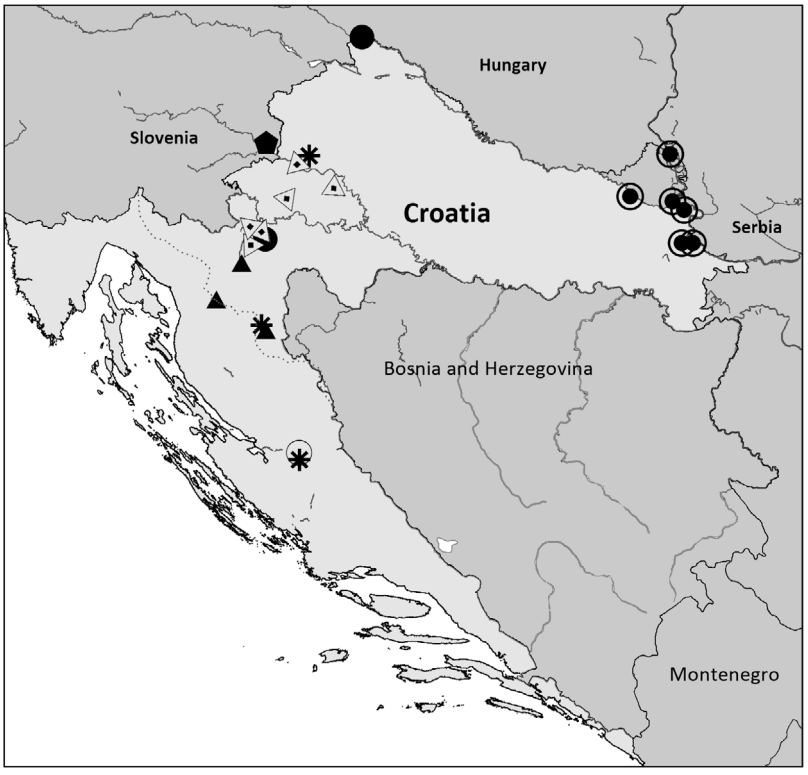

Fig. 1. Positions of studied populations (●: *Pacifastacus leniusculus*; ⦿: *Orconectes limosus*; ⬟: *Cherax quadricarinatus*; ✳: *Austropotamobius torrentium*; ○: *Austropotamobius pallipes*; ▲: *Astacus astacus*; ⧋: *Astacus leptodactylus*). Dotted line: border between the Black Sea and Adriatic Sea drainages

1 dead *Austropotamobius torrentium* specimen from a crayfish plague outbreak (Kraljevec Stream). Altogether, 165 individuals were tested (Table 1).

Detection of *Aphanomyces astaci* by quantitative real time-PCR

Sampled crayfish were stored in 96% ethanol. Tissue from one-half of the soft abdominal cuticle and 1 uropod was dissected from each crayfish using sterile instruments. Dissected tissues from each individual were placed in a single 1.5 ml tube, dried and stored in a deep-freezer at –80°C. Before further processing, 360 µl of ATL buffer from the DNeasy tissue kit (Qiagen) and 40 µl of Proteinase K solution were added to the dissected material. The mixture was then crushed by 1 scoop (ca. 50 µl) of stainless steel beads (1.6 mm diameter) using a BBX24B Bullet Blender (Next Advance) for 10 min at maximum speed. Buffer AL (400 µl) was added to the DNA mixture from the crushed cuticle; subsequently, the rest of the

Table 1. Sampling localities (if within the same water body, the name of the closest settlement is given in brackets) with coordinates (*x*, *y*; HTRS96 coordinate reference system) and numbers of samples (year of sampling in parentheses) per crayfish species

Crayfish species	Locality	*x*	*y*	No. of ind.
Astacus astacus	Mrežnica River	414660	5013432	1 (2011), 6 (2013)
Astacus astacus	Plitvice Lakes	429920	4971576	7 (2012), 7 (2013)
Astacus astacus	Stajnica River	398958	4990614	1 (2013)
Astacus leptodactylus	Crna Mlaka Lake	440360	5053896	1 (2009)
Astacus leptodactylus	Dobra River	417908	5037628	1 (2009)
Astacus leptodactylus	Jagodno Lake	472260	5062436	6 (2012), 3 (2013)
Astacus leptodactylus	Korana River (Ladvenjak)	428360	5031556	13 (2012), 11 (2013)
Astacus leptodactylus	Korana River (Šćulac)	422576	5024724	9 (2012)
Astacus leptodactylus	Rakitje Lake	447928	5073820	15 (2012)
Austropotamobius pallipes	Krupa River	450172	4894652	1 (2010), 20 (2013)
Austropotamobius torrentium	Kraljevec Stream	457088	5080832	1 (2012)
Austropotamobius torrentium	Plitvice Lakes	427265	4974619	3 (2012)
Austropotamobius torrentium	Zrmanja River	450840	4890556	1 (2010)
Cherax quadricarinatus	Čatež thermal oxbow lake (SI)	429792	5088864	6 (2012)
Orconectes limosus	Danube (Aljmaš)	691592	5046540	1 (2009)
Orconectes limosus	Danube (Batina)	682912	5081708	1 (2003)
Orconectes limosus	Danube (Vukovar)	697032	5025964	1 (2009), 8 (2011)
Orconectes limosus	Karišica River	657760	5055076	3 (2012)
Orconectes limosus	Kopački Rit	684308	5052140	3 (2003), 5 (2005)
Orconectes limosus	Vuka River	690088	5025884	2 (2012)
Pacifastacus leniusculus	Korana River	429200	5028436	12 (2012)
Pacifastacus leniusculus	Mura River	489776	5155072	16 (2011)

spin-column protocol of the DNeasy tissue kit was carried out.

Isolated material was then tested for the presence of *A. astaci* by the quantitative TaqMan MGB real-time PCR developed by Vrålstad et al. (2009), using the LightCycler® 480 Instrument (Roche). Experimental procedures were identical to those used by Filipová et al. (2013). Based on their PFU (PCR-forming units) values, samples were classified into semi-quantitative categories of pathogen load, ranging from A0 (no traces of *A. astaci* DNA) to A7 (an extremely high amount of *A. astaci* DNA in the sample), as proposed by Vrålstad et al. (2009).

Strain (genotype) determination

Nine discriminant microsatellite loci (*Aast* 2, 4, 6, 7, 9, 10, 12, 13, 14) developed by Grandjean et al. (2014) were genotyped on samples testing positively for *A. astaci* presence, with a level of infection of least A4 according to Vrålstad et al. (2009). All experimental procedures for microsatellite genotyping were identical to those described by Grandjean et al. (2014).

RESULTS

Results of qPCR confirmed the presence of *Aphanomyces astaci* in both native and non-native crayfish species in Croatia. Generally, tested samples from 3 ICS (*Astacus astacus*, *A. pallipes* and *A. torrentium*) showed a higher prevalence of infection (68, 38 and 40%, respectively) than *A. leptodactylus* (27%). Of the tested samples from 3 NICS, the highest infection prevalence was recorded in *Orconectes limosus* (58%) and less in *Pacifastacus leniusculus* (25%), while there were no infected individuals in the *Cherax quadricarinatus* population (Table 2).

Table 2. Number of *Aphanomyces astaci*-positive crayfish (from total number tested) per species

Species	No. infected/Total tested
Astacus astacus	15/22
Astacus leptodactylus	16/59
Astacus pallipes	8/21
Astacus torrentium	2/5
Cherax quadricarinatus	0/6
Orconectes limosus	14/24
Pacifastacus leniusculus	7/28

Details on the pathogen prevalence in different species populations are shown in Table 3. From the 3 *A. astacus* populations tested, *A. astaci* was detected in 2 (agent levels A2–A6), while in the Stajnica River we did not detect *A. astaci* in the single tested individual, so the infection status here remains unknown. In the 6 tested *A. leptodactylus* populations, the pathogen was not detected in 3 populations but in the other 3 its presence was recorded (agent levels A2–A6). Only 1 *A. pallipes* population was sampled in 2012 and 2013, and the presence of pathogen was recorded in 1 individual, sampled in 2012, with agent level A3. From 3 tested *A. torrentium* populations, the pathogen was recorded in 2. In one of them (Kraljevec Stream), crayfish was sampled at the end of the crayfish plague outbreak, and the determined agent level was A6, while, in the other (Zrmanja River), the agent level was A3. In the population from which the pathogen was not recorded (Plitvice Lakes), only 3 individuals were tested, so the infection status of the population remains unknown. From the 6 *O. limosus* populations studied, in 1 we tested only 2 individuals, and did not record the presence of the pathogen (Vuka River), and in 5 the agent was present, with an agent level from A2 to A3. Finally, in one of the studied *P. leniusculus* populations the pathogen was not recorded, while in the other the agent level varied from A2 to A5.

Genotyping from microsatellites detected the presence of a strain from Group A in the *A. astacus* and *A. torrentium* populations distributed in areas where NICS are not present, while in the sampled *A. pallipes* the agent level was too low for genotyping (Table 3). Interestingly, the genotype of *A. astaci* isolated from the Kraljevec Stream stone crayfish population at the end of the crayfish plague outbreak differed from a typical A (As) strain by 1 allele from the *Aast* 4 locus (Table 4).

The B (Ps I) strain was identified from the *A. leptodactylus* population that was in contact with *P. leniusculus* (Korana River, Ladvenjak) (Table 3). Surprisingly, in the gravel pit, Jagodno Lake samples of *A. leptodactylus* showed several microsatellite patterns mixed between SSR-A (As) and SSR-B (Ps) (Tables 3 & 4). Nomenclature refers to that commonly used for microsatellite genotypes (simple sequence repeats, SSR) and was adopted from Grandjean et al. (2014).

The agent level, when positive, in non-native crayfish was generally low. It reached the A5 level in just 1 *P. leniusculus* individual, for which the genotype was determined as SSR-B with a microsatellite pattern identical to that of the reference (Ps I) strain (Tables 3 & 4) except at 2 loci (*Aast* 9 and *Aast* 10). The agent level in the studied *O. limosus* populations was too low for genotyping.

Table 3. Details on crayfish species, year of sampling, number of tested crayfish, agent level and genotypes of *Aphanomyces astaci* (named according to Grandjean et al. 2014) populations recorded in different localities. Only samples with at least agent level A4 were used for microsatellite genotyping (characters in **bold**). ICS: indigenous crayfish species; NICS: non-indigenous crayfish species

Population	Crayfish species	Year	n	Agent level (no. of ind.)	*A. astaci* genotype
Infection status of ICS populations					
Kraljevec Stream[a]	*Austropotamobius torrentium*	2012	1	**A6 (1)**	**SSR-A_3**
Plitvice Lakes		2012	3	A0 (3)	
Zrmanja River		2010	1	A3 (1)	
Plitvice Lakes	*Astacus astacus*	2012	7	A0 (1), A1 (1), A2 (1), A3 (3), **A4 (1)**	**SSR-A_2**
		2013	7	A0 (3), A1 (2), A2 (1), **A4 (1)**	**SSR-A_2**
Mrežnica River		2011	1	**A6 (1)**	**SSR-A_2**
		2013	6	A0 (2), A1 (2), A2 (1), **A5 (1)**	**SSR-A_2**
Stajnica River		2013	1	A0 (1)	
Krupa River	*Austropotamobius pallipes*	2010	1	A3 (1)	
		2013	20	A0 (13), A1 (7)	
Korana River (Ladvenjak)	*Astacus leptodactylus*	2012	13	A0 (12), **A4 (1)**	**SSR-B**
		2013	11	A0 (4), A1 (3), A2 (1), A3 (1), **A4 (1), A6 (1)**	**SSR-B**
Jagodno Lake		2012	6	A0 (2), **A4 (4)**	**SSR-AB**
		2013	3	A3(2), **A4 (1)**	**SSR- AB**
Crna Mlaka Lake		2009	1	A3 (1)	
Rakitje Lake		2012	15	A0 (15)	
Korana River (Šćulac)		2012	9	A0 (9)	
Dobra River		2009	1	A0 (1)	
Infection status of NICS populations (*A. astaci* carriers)					
Kopački Rit	*Orconectes limosus*	2003	3	A3 (3)	
		2005	5	A3 (5)	
Danube (Vukovar)		2009	1	A3 (1)	
		2011	8	A0 (7), A1 (1), A2 (1)	
Danube (Aljmaš)		2009	1	A3 (1)	
Danube (Batina)		2003	1	A3 (1)	
Karašica River		2012	3	A0 (2), A2 (1)	
Vuka River		2012	2	A0 (2)	
Korana River	*Pacifastacus leniusculus*	2012	12	A0 (12)	
Mura River		2011	16	A0 (9), A2 (4), A3 (1), A4 (1), **A5 (1)**	**SSR-B_2**
Čatež thermal oxbow lake (SI)	*Cherax quadricarinatus*	2012	6	A0 (6)	

[a]Crayfish collected after the crayfish plague outbreak

DISCUSSION

Historical sources (Lindes 1884, Anonymous 1897, 1899, 1901) mention crayfish mass mortalities in Croatia caused by crayfish plague outbreaks. Moreover, the precise date (11 November 1880) of the first outbreak in Croatia was recorded (Anonymous 1901). All of those historical records emphasise that crayfish plague devastated native crayfish populations of continental Croatia (the Drava and Sava drainages), while isolated populations of the Alpine and Mediterranean regions (Lika, Gorski Kotar) remained plague-free. Furthermore, records point out that native crayfish species, from Croatian populations that were considered unaffected by crayfish plague, were used to restock water bodies in Austria, Germany and Switzerland, where crayfish plague had decimated entire populations (Anonymous 1899).

According to Plančić (1973), another crayfish plague outbreak hit Croatian crayfish populations at the end of 1960s and, since then, no records of the disease in Croatia exist. Hence, this is the first study on the prevalence of the pathogen in Croatia. Results of our research, contrary to our expectations, indicated evidence of *Aphanomyces astaci* in all of the studied species, except *Cherax quadricarinatus* (extra samples from Slovenia analysed in this study) (Table 3).

Table 4. Allele sizes of 9 *Aphanomyces astaci* simple sequence repeat (SSR) loci from pure strains (*A. astaci* Groups A to E as in Grandjean et al. 2014 with codes below) and those obtained from infected crayfish populations used in this research (locality and strain below species names). –: not determined

SSR locus	*Aphanomyces astaci* group					Crayfish species used in this research					
	A	B	C	D	E	*Pacifastacus leniusculus*	*Austropotamobius torrentium*		*Astacus leptodactylus*		
	VI03557	VI03555	VI03558	VI03556	Evira4805	Mura B[a]	Kraljevec A[a]	Korana B	Jagodno A & B		
									2012		2014
Aast 2	160	142	154	138	150	142	160	142	142/160[a]	142	142
Aast 4	103	87	87	131	87/89	87	87[a]	87	103	87	103
Aast 6	157	148	148	148	148/157	148	157	148	157	148	148
Aast 7	207	215	191	203	207	215	207	215	207	215	215
Aast 9	180	164/182	164/168	180	168/182	164/180[a]	180	164	180	164/182	164/182
Aast 10	142	132	132	142	132/142	132/138[a]	142	132	132/142[a]	132/138	138
Aast 12	–	226/240	226	234	234/240	226/240	–	226/240	–	226/240	226/240
Aast 13	194	202	202	194	194/202	202	194	202	194	194/202[a]	194
Aast 14	246	248	248	250	248	248	246	248	246/248[a]	248	248

[a]Observed differences from typical strains (both populations and alleles)

Surprisingly, the pathogen was found even in the populations of *Austropotamobius pallipes* and *A. torrentium* that were isolated in rivers of the Adriatic Sea drainage. Those rivers have no surface connections to water bodies in continental Croatia, where non-native carriers of the pathogen exist today or crayfish plague outbreaks have been recorded in the past (Fig. 1, Table 3). Chronically infected, viable populations of *A. torrentium* have been recently recorded in Slovenia, in the Sava River drainage (Kušar et al. 2013). In their research Kušar et al. (2013) did not record the presence of the pathogen in *A. pallipes* in the Adriatic Sea drainage; thus, as far as we are aware, our record is the first one for a viable, chronically infected population of this species in Europe. Additionally, the level of infection in viable *A. torrentium* and *A. pallipes* populations from the Adriatic drainage was too low for genotyping.

However, the genotype of *A. torrentium* sampled at the end of a crayfish plague outbreak in an isolated stream on the Medvednica Mountain (Sava River drainage) differed from a typical A (As) strain in 1 allele. Variation within the A (As) genotype was already detected in studies of the *A. astaci* chitinase gene (Makkonen et al. 2012), and the recent research by Grandjean et al. (2014) points to the prospect of variation detection among and within the crayfish plague strains. However, as the specificity of microsatellite amplifications for *A. astaci* has been tested from only 2 *Aphanomyces* species, cross amplifications could occur from other species of the *Aphanomyces* genus not investigated in their study. Hence, the obtained results require further sampling and research in order to clarify the relationship between the Medvednica genotype and typical A (As) strains, while bearing in mind that application of a new marker system must be carried out with caution (Grandjean et al. 2014). Nevertheless, at this point, we can hypothesise that the observed difference may be a consequence of the fast evolution of the pathogen, which was brought to Europe by an unknown host (Diéguez-Uribeondo et al. 2006, Kozubíková et al. 2011a), as it is known that a host–parasite relationship is an active, fast and mutually adaptive interaction (Van Baalen 1998). Under normal living conditions, North American crayfish species are resistant to their strains (B [PsI], C [PsII], D [Pc]), E [Or]), while European species are, to some extent, resistant to the old A (As) strain. It could be speculated that European crayfish species developed resistance to the A (As) strain or their resistance may be a consequence of lowered virulence of the pathogen (Viljamaa-Dirks et al. 2015). Either of these sce-

narios could explain the existence of viable chronically infected populations of ICS across Europe (Jussila et al. 2011, Svoboda et al. 2012, Kušar et al. 2013, present study).

Similarly to other studies (Jussila et al. 2011, Kokko et al. 2012, Pârvulescu et al. 2012, Svoboda et al. 2012, Makkonen et al. 2014) we have found chronic infection in the *A. astacus* and *A. leptodactylus* populations (Table 3). While populations of *A. astacus* are infected with the European A (As) genotype, populations of *A. leptodactylus* are infected with the SSR-B (Ps) genotype (Korana River–Ladvenjak) or with mixed patterns from both SSR-A (As) and SSR-B (Ps) genotypes (Jagodno Lake). Record of the B (Ps) strain in *A. leptodactylus* from the Korana River was expected, since it co-occurs in the lower section of the river harbouring a *P. leniusculus* population that is actively expanding its range (Hudina et al. 2013). The record of several genotyping patterns mixed between both strains from Groups A and B in an isolated population in Jagodno Lake was surprising. It was expected that *A. leptodactylus*, which coexists in the Korana River with *P. leniusculus*, would carry the SSR-B (Ps) strain. The finding of mixed strains is probably the consequence of repetitive introduction of *A. leptodactylus* and *A. astacus* from different sources into the lake (authors' pers. comm. with local inhabitants). Specimens of *A. astacus* were introduced from an isolated gravel pit; those crayfish have not been tested for pathogen presence, but we could speculate that they carried the SSR-A (As) strain. Specimens of *A. leptodactylus* were introduced to the lake from the Mrežnica River, from a locality close to its confluence with the Korana River, which is inhabited by *P. leniusculus*. Consequently, it is possible that *A. leptodactylus* sampled for introduction were infected with the SSR-B (Ps) strain. This scenario would explain the presence of the 2 *A. astaci* genotypes in tested individuals. The microsatellite patterns observed in *A. astaci* from the infected animals in Jagodno Lake are complex and could indicate recombination events between the 2 strains, because only a few loci show alleles from both strains (e.g. *Aast* 14: 246 [SSR-A] / 248 [SSR-B]). Sexual reproduction of *A. astaci* has been reported by Rennerfelt (1936), but his research was probably conducted on mixed cultures; thus, observed sexual reproduction did not necessarily refer to *A. astaci*. In addition, the existence of sexual reproduction has not been confirmed in recent studies on pure cultures (Söderhäll & Cerenius 1999, Diéguez-Uribeondo et al. 2009). However, different reasons could be advanced to explain the obtained results, for example, that (1) both strains were present in the same individuals because some alleles could be better amplified than others, (2) some allele may have been amplified from an unforeseen cross-reaction (other oomcycetes present in the sample) and (3) true variation occurred in the strain. Further genomic analyses on the strains mentioned are necessary to resolve this interesting finding. Finally, a new SSR-B genotype was reported for *P. leniusculus* from the Mura population differing in allele composition from 2 loci; this is probably the result of several introductions of specimens from different parts of their natural range in Europe, as reported in several studies on population genetics (Grandjean & Souty-Grosset 1997, Froufe et al. 2015).

In summary, the first study of *A. astaci* occurrence in Croatia showed that the pathogen is widespread in both ICS and NICS populations. Discovery of the B (Ps) strain in *P. leniusculus* and *A. leptodactylus* occupying the same water body was somewhat expected, while the presence of the pathogen in isolated *A. torrentium* and *A. pallipes* populations from the Adriatic Sea drainage, as well as the presence of a slightly different A (As) strain in the isolated *A. torrentium* population and the presence of 2 different strains in *A. leptodactylus* represent unexpected and intriguing results.

Further systematic and continuous studies with the application of new molecular techniques, especially on isolated ICS populations, are needed in order to obtain clear insight on the pathogen's spread, diversity and origin. Future results will be applicable in the development of effective management plans aiming to protect vulnerable native species in Croatia, as well as to scan potential plague-free sites for future ICS reintroduction.

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