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Hidden sites in the distribution of the crayfish plague pathogen *Aphanomyces astaci* in Eastern Europe: relicts of genetic groups from older outbreaks?

Jörn PANTELEIT[a], Nina Sophie KELLER[a,1], Javier DIÉGUEZ-URIBEONDO[b], Jenny MAKKONEN[c], Laura MARTÍN-TORRIJOS[b], Viorica PATRULEA[d] Mălina PÎRVU[e], Cristina PREDA[f], Anne SCHRIMPF[a], and Lucian PÂRVULESCU[e]

[a]University of Koblenz-Landau, Institute for Environmental Sciences, 76829 Landau, Germany

panteleit@uni-landau.de; nina-sophie.keller@ufz.de; schrimpf@uni-landau.de

[b]Real Jardín Botánico CSIC, Departamento de Micología, 28014 Madrid, Spain

dieguez@rjb.csic.es; lmtorrijos@rjb.csic.es

[c]Department of Biology, University of Eastern Finland, 70211 Kuopio, Finland

jenny.makkonen@uef.fi

[d]University of Geneva, University of Lausanne, School of Pharmaceutical Sciences, 1211 Geneva, Switzerland

viorica.patrulea@unige.ch

[e]West University of Timisoara, Faculty of Chemistry, Biology, Geography, Department of Biology-Chemistry, 300115 Timisoara, Romania

lucian.parvulescu@e-uvt.ro; malina.pirvu@e-uvt.ro

[f]Ovidius University of Constanta, Faculty of Natural and Agricultural Sciences, 900470 Constanta, Romania

preda_cristina2006@yahoo.com

[1]Current address: Helmholtz-Centre for Environmental Research (UfZ), Department of

Isotope Biogeochemistry, Permoserstrasse 15, 04318 Leipzig, Germany

Corresponding authors: **1)** Jörn PANTELEIT; e-mail: panteleit@uni-landau.de; phone: +49 6341 280 31349 **2)** Lucian PÂRVULESCU; e-mail: lucian.parvulescu@e-uvt.ro; phone: +40 76 648 9256

Abstract:

The crayfish plague agent *Aphanomyces astaci* is one of the world's most threatening invasive species. Originally from North America, the pathogen is being imported alongside American crayfish species, which are used for various purposes. In this study, we investigated the marginal, currently known distribution area of the pathogen in Eastern Europe by sampling narrow-clawed crayfish (*Astacus leptodactylus)* and spiny-cheek crayfish (*Orconectes limosus*) populations. In addition, using specific real-time PCR, we tested several marine decapod species, which also occur in brackish waters of the Danube at the West coast of the Black Sea and the Dniester River basin. By sequencing the nuclear chitinase gene, mitochondrial rnnS/rnnL DNA and by genotyping using microsatellite markers, we identified the *A. astaci* haplogroups of highly infected specimens. The *A. astaci* DNA was detected in 9% of the investigated *A. leptodactylus* samples, both in invaded and non-invaded sectors, and in 8% of the studied *O. limosus* samples. None of the marine decapods tested positive for *A. astaci*. The results revealed that narrow-clawed crayfish from the Dniester River carried the *A. astaci* B-haplogroup, while *A. astaci* from the Danube Delta belonged to the A- and B-haplogroups. In the invaded sector of the Danube, we also identified the A-haplogroup. Microsatellite analysis revealed a genotype identical to the genotype Up. It might be that some of the detected *A. astaci* haplogroups are relics from older outbreaks in the late 19th century, which may have persisted as a chronic infection for several decades in crayfish populations.

Keywords: Danube; Dniester River; invasive species; narrow-clawed crayfish; haplotypes; real-time PCR

1. INTRODUCTION

Usually, the relationship between a parasite and its host is in a natural equilibrium, which ensures the survival of both species under certain conditions (Anderson and May 1982). This relationship is influenced by the environment, the host immunity, and the parasite's virulence. If both the parasite and the host are translocated to a new environment, the host may benefit from the parasite's ability to also infect and weaken competitors of the original host (García-Ramos et al. 2015), as these may lack an evolutionary adaptation to the parasite. This may significantly increase the dominance of a natural host towards newly infected competitors (Strauss et al. 2012). The last century came with one of the most notable disasters for crayfish wild populations in Europe with episodes of mass mortalities driven by the crayfish plague (Alderman 1996; Lowe et al. 2004), a disease caused by the invasive oomycete *Aphanomyces astaci* SCHIKORA, 1903. While the pathogen's North American species specific origin is well known, as revealed by Unestam (1972) and by molecular analysis (Huang et al. 1994; Diéguez-Uribeondo et al. 1995; Makkonen et al. 2012a), its past and current distribution in Europe remains to be studied. This is especially true in boundary areas or regions not invaded by North-American crayfish species.

Aphanomyces astaci has coevolved with North American crayfish species (Unestam 1972; Söderhäll and Cerenius 1992). As a result, American crayfish do not usually die due to an infection with *A. astaci* (Cerenius et al. 2003). On the contrary, European crayfish species are highly susceptible and an infection usually leads to a quick death (Unestam 1969a; Alderman et al. 1987; Cerenius et al. 2009). Recently, it was shown that other freshwater crustaceans, specifically, the crab species *Eriocheir sinensis* MILNE EDWARDS, 1853 and *Potamon potamios* (OLIVIER, 1804) (Schrimpf et al. 2014; Svoboda et al. 2014) can serve as vectors for *A. astaci*.

Due to its virulence and devastating effects on indigenous crayfish species all over the

world, *A. astaci* was classified among the world's 100 worst invasive alien species (Lowe et al. 2004). The first report regarding crayfish plague in Europe originates from the late 1850s, before the first documented introductions of North American crayfish species (Cornalia 1860). Alderman (1996) estimated the distribution of this disease based on the records of mass mortalities associating them with crayfish plague outbreaks. Repeated outbreaks were noted in the late 19th and early 20th centuries across substantial parts of Europe including the West, Central and East European to Eurasian river basins (Alderman 1996).

Based on RAPD-PCR analysis, five *A. astaci* genetic groups have been identified to date, which can be assigned to different host species according to their origins. Due to the multiple introductions of non-indigenous crayfish species and aggressive successful spreading of several species across Europe (Kouba et al. 2014), the current distribution of *A. astaci* is a mosaic of different genetic groups (Svoboda et al. 2016). RAPD-groups B and C were found on signal crayfish, *Pacifastacus leniusculus* (DANA, 1852) (Huang et al. 1994), group D on red swamp crayfish, *Procambarus clarkii* (GIRARD, 1852) (Diéguez-Uribeondo et al. 1995) and group E was identified on spiny-cheek crayfish, *Orconectes limosus* (RAFINESQUE, 1817) (Kozubíková et al. 2011). In contrast to these RAPD-groups, the original host of group A that was isolated from an indigenous European crayfish, i.e., *Astacus astacus* (LINNAEUS, 1758) and *Astacus leptodactylus* (ESCHSCHOLTZ, 1823), is still unknown (Souty-Grosset et al. 2006; Makkonen et al. 2012a; Viljamaa-Dirks et al. 2013). These RAPD-groups are often referred to as As, PsI, PsII, Pc, and Or. However, in this manuscript we will use the original lettering, A-E. The most recent development of primers targeting the mitochondrial small and large ribosomal subunits (rnnS and rnnL) divided the known RAPD-groups into four haplogroups (A, B, D and E) (Makkonen et al. 2018). The A-haplogroup contains the *A. astaci* RADP-PCR groups A and C, the B-haplogroup the strains from *A. astaci* RADP-PCR groups B, the D-haplogroup contains the d1 and d2-haplotypes from *A.*

astaci RADP-PCR groups D, and the E-haplotype contains the strains from *A. astaci* RADP-PCR group E.

Moreover, differences in virulence were measured between groups (Diéguez-Uribeondo et al. 1995; Makkonen et al. 2012b; Jussila et al. 2013; Viljamaa-Dirks et al. 2016). In laboratory experiments, group B isolates caused the rapid and total mortality of noble crayfish *A. astacus*, while group A was less virulent in general (Makkonen et al. 2012b; Becking et al. 2015). Although European crayfish species are generally highly susceptible and usually die within a few days after infection, evidences for an evolutionary adaption of host and pathogen seem to be the reason for the survival of some crayfish populations. This has been shown for *A. astacus* (Jussila et al. 2011; Viljamaa-Dirks et al. 2011; Makkonen et al. 2012b; Viljamaa-Dirks et al. 2013) and *Austropotamobius pallipes* (LEREBOULLET, 1858) (Martin-Torrijos et al. 2017). Moreover, pathogen persistence in indigenous European crayfish species has also been described for the narrow-clawed crayfish *A. leptodactylus* (ESCHSCHOLTZ, 1823) (Kokko et al. 2012; Svoboda et al. 2012; Schrimpf et al. 2012) and in the stone crayfish *Austropotamobius torrentium* (SCHRANK, 1803) (Kušar et al. 2013; Jussila et al. 2017).

Additionally, another technique that uses microsatellite markers allows the further identification of known genotypes of *A. astaci* not only from pure cultures, but also from infected crayfish tissue, which can be assigned to the different genetic groups. One group can consist of different genotypes, e.g., genetic group A, which contains at least the microsatellite genotypes A_1 and A_2 (Grandjean et al. 2014). The finer discrimination makes genotyping an important tool for the characterization of *A. astaci*, and may, in some cases, allow a reconstruction of the origin of one specific crayfish plague occurrence.

Range extensions of American crayfish species pose a permanent threat to indigenous crayfish (Holdich et al. 2009), because they can act as chronic *A. astaci* reservoirs

(Kozubíková et al. 2009). One of the most invasive crayfish species and also carrier of *A. astaci* is *O. limosus*. This species has been documented in Europe since the 1890s (Souty-Grosset et al. 2006; Filipová et al. 2011). The reasons for the high invasive potential and spreading of *O. limosus* in Europe are higher fecundity and faster egg development in comparison to European species (Kozák et al. 2006; Souty-Grosset et al. 2006; Pârvulescu et al. 2015), as well as a wide habitat range and tolerance towards unfavorable environmental conditions, e.g., drought, cold, and low water quality (Souty-Grosset et al. 2006; Holdich and Black 2007). Although coexistence with European crayfish species, i.e., *A. astacus*, *A. leptodactylus* (Schrimpf et al. 2013a), white-clawed crayfish *A. pallipes* (Caprioli et al. 2013) and *A. torrentium* (Kušar et al. 2013) was noted, *O. limosus* mainly replaced indigenous crayfish species in Europe (Souty-Grosset et al. 2006).

For the first time, *A. astaci* was detected in the Romanian Danube River in 2011, specifically, in *A. leptodactylus* populations coexisting with the invasive *O. limosus*. Surprisingly, the pathogen was also confirmed in *A. leptodactylus* populations around 70 km downstream of the *O. limosus* invasion front (Pârvulescu et al. 2012). In 2012, the pathogen was also detected in one of the three branches of the Danube Delta, the Chilia Channel (Schrimpf et al. 2012). It is still unknown whether marine decapod species, which are highly abundant in the brackish waters of the Danube Delta (Petrescu et al. 2010), can also act as vectors of *A. astaci*. The Danube Delta is a highly protected area where crayfish mass mortalities have not been reported to date. Due to the absence of *O. limosus* or other North American species in the region, the origin of *A. astaci* remained unknown. This study aims to extend the knowledge of the *A. astaci* distribution and genetic group assignment by investigating the estimated eastern range of the pathogen's distribution area. In addition to crayfish species, different marine decapod species were tested for a crayfish plague infection. Furthermore, in the case of *A. astaci* occurrence, we intended to identify the genetic group of

crayfish plague pathogen by means of sequence analyses as well as microsatellite analysis.

2. METHODS

2.1 Crayfish and marine decapods sampling

In 2015, we collected samples from indigenous *A. leptodactylus* populations in the Danube River as well as invaded populations of *A. leptodactylus* mixed with non-indigenous *O. limosus*. (Fig. 1; Table 1). In addition to the existing material from the Chilia Channel in the Danube Delta, 58 samples from the lake complex Rosu - Puiu - Lumina in the Danube Delta were collected in spring 2016. Outside of the Danube catchment, 104 *A. leptodactylus* were sampled in the Dniester River, Republic of Moldova, near Dubăsari in late spring between 2013 and 2015. Crayfish were captured by trapping or by fishing nets. All the samples consisted of soft abdominal cuticle, walking legs, telson and parts of the uropods stored in 96% ethanol. In addition to the newly collected and tested samples, we also include samples from an earlier publication, including 37 *A. leptodactylus* specimens from the Danube Delta that were tested for an infection with *A. astaci* (Schrimpf et al. 2012).

In order to test the hypothesis whether marine decapods, which can also appear in brackish waters, act as crayfish plague vectors, seven of the most common species were sampled at the western coast of the Black Sea, near Agigea (44°04′32″N/28°46′37″E) and Năvodari (44°18′48″N/28°49′27″E) (Fig. 1, Table 2). The samples included nine individuals of the rockpool shrimp, *Palaemon elegans* RATHKE, 1837, eight individuals of the jaguar round crab, *Xantho poressa* (OLIVI, 1792), the marbled crab, *Pachygrapsus marmoratus* (FABRICIUS, 1787), and the flying crab, *Liocarcinus holsatus* (FABRICIUS, 1798). Further, two individuals of the bristly crab, *Pilumnus hirtellus* (LINNAEUS, 1761), and one specimen of each the dwarf crab, *Rhithropanopeus harrisii* (GOULD, 1841) and the porcelain crab, *Pisidia longimana* (RISSO, 1816) were captured by hand in the summer months between 2013 and

2014. Pieces of the ventral carapax cuticle, abdomen and walking legs were dissected and stored in 96% ethanol.

2.2 Real-time PCR

DNA was extracted using a CTAB-method according to Vrålstad et al. (2009). To assess the infection status of crayfish and marine decapods, we conducted a TaqMan® minor groove binder (MGB) qPCR, targeting the ITS region, as described in Vrålstad et al. (2009), with some modifications (Schrimpf et al. 2013b). Infection status and agent levels were defined according to Vrålstad et al. (2009).

The proportions of infected crayfish in various river sectors were compared using Fisher exact tests. The 95% confidence intervals of infected specimens were calculated using the software RStudio V.1.0.44 (R Core Team 2016) with the package epiR V.0.9 (Stevenson et al. 2016).

2.3 Sequence analyses

If possible, the haplotype of *A. astaci* was identified for infected samples with high agent levels of A3, i.e., a PFU of at least 800, and above using sequence analysis of three different genes: the nuclear chitinase gene, and the mitochondrial ribosomal rnnS and rnnL subunits. The rnnS/rnnL mitochondrial haplogroup A contains RAPD-groups A and C. Use of the chitinase sequences allows for the discrimination of the two RAPD-groups A and C. First, we sequenced a 370 base pair (bp) long DNA fragment of the nuclear chitinase gene according to Makkonen et al. (2012a), with some modifications. The modifications were as follows: we used 5x PCR buffer, 0.025 U TaqMan® Taq (both Promega, Mannheim, Germany), 2 mM $MgCl_2$, 0.2 mM dNTP mix (both Fermentas, St. Leon-Rot, Germany), 0.2 µM primers AAChiF and AAChiR and added 3 µl DNA template for a final volume of 25 µl. The Two

other genes were the mitochondrial ribosomal rnnS (512 bp) and rnnL (435 bp) subunits, according to Makkonen et al. (2018). Primer AphSSUF (5'-GGGCGGTGTGTACAAAGTCT-3'), AphSSUR (5'-AGCACTCCGCCTGAAGAGTA-3'), AphLSUF (5'AGGCGAAAGCTTACTATGATGG-3'), and AphLSUR (5'-CCAATTCTGTGCCACCTTCT-3') were used in the following reactions: The PCR reaction mixture contained 0.4 µM of each primer, 0.75X Dream*Taq* Green master mix (Thermo Fisher Scientific), 0.5 U Dream*Taq* DNA polymerase (Thermo Fisher Scientific), 0.17 mM dNTPs, and 2.5 µl of the DNA template. The mixture was filled up to 12.5 µl with PCR-grade water. PCR was carried out on a Primus 96 Plus Thermal Cycler (PEQLAB Biotechnologies GmbH) with the following conditions: 95°C, 3 min, 30x (95°C, 30 s; 60°C, 30 s; 72°C, 30 s), and 72°C 10 min. Each run contained a positive control (*A. astaci* DNA) and a blank reaction without a template. The amplification was checked on an agarose gel with EtBr labelling. Sequence analysis was also conducted for one sample with a high agent level (A6) from an earlier study (Schrimpf et al. 2012), because the haplotype of this highly infected sample had previously not been identified. The positive control consisted of DNA isolated from a pure culture of *A. astaci* strain UEF_SATR1, RAPD PCR group B and the negative control of pure reaction master mix. PCR products were sequenced on a 3730 DNA Analyzer eight capillary sequencer (Applied Biosystems, MA, USA) by the company Seq IT GmbH & Co.KG (Kaiserslautern, Germany). All sequences were compared with pure cultured reference strains of *A. astaci*. The reference strains of pure culture isolates for RADP-PCR group A (UEF-AT1D, Jussila et al. 2017) and B (UEF-SATR1, Jussila et al. 2013) were obtained from the University of Eastern Finland, Kuopio campus, Finland. The reference strain Kv1 (RADP-PCR group C) was from Sweden (Huang et al. 1994) and reference strain AP03 (RADP-PCR group D) from Spain (Rezinciuc et al. 2014). Reference sequences of the RADP-PCR group E were generated from pure culture samples of the

strains Li05 and Li08, isolated from *A. astacus* which had inhabited the Litavka stream (Kozubíková-Balcarová et al. 2013). The sequences were aligned and edited with the program Geneious R7 (http://www.geneious.com , Kearse et al. 2012).

2.4 Microsatellite analysis

We conducted microsatellite analysis using nine co-dominant microsatellite markers according to Grandjean et al. (2014) for samples with high agent levels of A3, i.e., a PFU of at least 800, and above. Only one sample was successfully analyzed with microsatellite analysis, sample DC18 from the Danube Delta. The other samples most likely did not contain enough *A. astaci* DNA for successful Microsatellite analysis. Amplification was done in two batches using the QIAGEN Multiplex PCR Kit (QIAGEN, Netherlands) according to the manufacturer's instructions, with 0.25 µl each of the labeled primers added Aast4, Aast6, Aast7, and Aast14 for Batch A, and Aast2 Aast9, Aast10, Aast12, and Aast13 for Batch B. Then, 1 µl DNA template was added for a final volume of 5 µl and 5.5 µl, respectively. PCR grade water was used as a negative control. PCR conditions were as follows: 95°C for 2 min, 35 cycles of 95°C for 30 s, 54°C for 90 s and 72°C for 60 s. The final elongation step was at 72°C for 5 min. PCR conditions were the same for both batches and PCR was performed on a Primus 96 Plus Thermal Cycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany)

For the fragment analysis, 0.5 µl PCR product was mixed with 27.2 µl SLS buffer and 0.3 µl 400 bp standard (Beckman Coulter, Brea CA, United States). Analyses were conducted on a Beckman Coulter CEQ 8000 eight capillary sequencer. Alleles were scored using the GenMarker software (version 1.95, SoftGenetics LLC) and compared to reference genotypes from pure cultures. The reference genotypes were created from the same reference cultures as described in section 2.3 and additionally a mixed DNA sample (Up4) was used as a reference for genotype Up. This sample was from a crayfish plague outbreak in the Uporsky Brook, Czech Republic.

3. RESULTS

Overall, 25 out of the 266 (9%) *A. leptodactylus* samples tested positive via real-time PCR for *A. astaci* DNA (Table 3). Agent levels of the infected samples ranged from A2 to A6. Of the 38 *O. limosus* samples, 3 (8%) tested positive, all with agent level A2. Analysis revealed that 6 of 104 (6%) *A. leptodactylus* from the Dniester River were positive, showing very low (A2) to moderate (A4) levels of *A. astaci* DNA. The *A. astaci* infection prevalence among tested *A. leptodactylus* from the invaded part of the Danube was 9 out of 43 (21%), whereas no *A. astaci* DNA was detected in the individuals tested from the non-invaded sector of the Danube River. In the Danube Delta, one sample had very high DNA amounts (A6) of *A. astaci*. In the Delta, 10 out of the 58 (17%) tested samples contained at least low (A2) amounts of *A.* astaci DNA. The Fisher tests showed that the proportions of infected crayfish were not significantly different for *A. leptodactylus* and *O. limosus* in the invaded part of the Danube ($p = 0.125$). There were no statistically significant differences between proportions of infected *A. leptodactylus* in the invaded Danube and the Danube Delta ($p = 0.797$). Nevertheless, the proportion of infected *A. leptodactylus* was significantly greater in the invaded part of the Danube and in the Danube Delta compared to Dniester River ($p = 0.013$ and $p = 0.027$, respectively). No DNA of *A. astaci* could be detected in any marine decapod sample. Finally, no significant differences ($p = 0.205$) were found comparing the proportions of infected *A. leptodactylus* in the Danube Delta in the present study to those recorded in the previous study by Schrimpf et al. (2012).

Sequence analyses of three genes (chitinase, rnnL and rnnS) were successful for four samples: the sample with agent level A6 (Sample ID DC18) collected from the Chilia Channel in 2012, the northern branch of the Danube in the Delta; two samples from the invaded part of the Danube River in Serbia with agent level A3 (Sample IDs ASLSRB 35 and

58); and one sample collected from the lake complex in the southern Danube Delta in 2015 with agent level A6 (Sample ID DD108) (GenBank accession numbers: MF740801–MF740809 and MF774441–MF774443). Two different *A. astaci* haplogroups were detected. The A-haplogroup was detected in the samples from the Chilia Channel in the Danube Delta (DC18) and in the invaded part of the Danube River (ASLSRB35/58) and the B-haplogroup from samples collected from the Roşu - Puiu - Lumina lake complex in the Danube Delta (DD108) (Table 4). The four chitinase sequences were identical to each other. They corresponded to those of RAPD-groups B, C and E. These groups cannot be distinguished by the chitinase gene alone. Combining the results from the chitinase gene and the mitochondrial rnnL and rnnS genes allowed the haplogroup A samples, from the Danube Delta and the Danube River in Serbia, to be more closely identified as RAPD-group C, since the chitinase sequences were identical to those of RAPD-group C (Table 4). One of the *A. leptodactylus* samples with agent level A4 collected from the Dniester River was successfully sequenced only for the mitochondrial rnnL gene. This allowed the sample to be assigned to the *A. astaci* B-haplogroup (Sample ID LP_AlD26).

Microsatellite analysis was only successful for the sample with agent level A6 from the Chilia Channel in the Danube Delta collected already in 2012 (DC18). For this sample, loci Aast 2, Aast 7, and Aast 10 were heterozygous, while the remaining 6 loci showed homozygosity for alleles (Appendix A). The allele pattern was identical to the Up-genotype found in the Czech Republic (Grandjean et al. 2014).

4. DISCUSSION

Of the 304 crayfish samples collected from the marginal distribution of the invasive crayfish species *O. limosus* in the Danube, and also a non-invaded basin in the eastern range of estimated historical *A. astaci* distribution, the Dniester River, 28 of the samples tested

positive for the crayfish plague agent *A. astaci*, specifically, 25 indigenous *A. leptodactylus* samples and 3 non-indigenous *O. limosus* samples. To extend the results further, this data is supplemented by additional *A. leptodactylus* samples (11 positive out of 37) from the Danube Delta, which had already been analyzed by Schrimpf et al. (2012). Our results show a low *A. astaci* infection prevalence of *A. leptodactylus* populations in both the absence and presence of the North American crayfish species *O. limosus*. The infection prevalence in *A. leptodactylus* is similar to earlier studies, where apparently healthy *A. leptodactylus* populations were found to be infected with *A. astaci* (e.g. Maguire et al. 2016; Kokko et al. 2018). In comparison to data from the Danube in 2012, the infection prevalence was a little lower (Pârvulescu et al. 2012), which could however be attributed to natural fluctuations of infection prevalence, like it was for example observed for *O. limosus* (Matasová et al. 2011). The reasons for the low infection prevalence in the Dniester River as well as the Danube River (6% and 20%, respectively), in general, remain unclear. One possibility might be that the indigenous *A. leptodactylus* populations are, to some degree, resistant to *A. astaci* (Unestam 1969b; Alderman et al.1987; Kokko et al. 2012, 2018; Maguire et al. 2016), which may result in reduced agent levels and the low prevalence of *A. astaci* in crayfish populations (Cerenius et al. 2003). The infection prevalence was similar to those of resistant North American crayfish, e.g., *P. leniusculus* (Filipová et al. 2013).

Applying sequence analysis, we identified the B-haplogroup (RAPD-PCR group B) in the Dniester River (Sample ID LP_AlD_26), Moldova, as well as in a sample from the Danube Delta in Romania. The finding of the B-haplogroup in the geographically separated Dniester River, i.e., an area where no *O. limosus*, or any other North American crayfish species, are present yet, supports the hypothesis that the infection is chronic to the tested *A. leptodactylus* populations. Chronically infected *A. leptodactylus* populations in Turkey showed an increased tolerance (balanced host-pathogen relationship), after the recovery of a

crayfish plague collapse in the mid-1980s, due to lowered virulence of *A. astaci*, increased resistance of crayfish, or both (Kokko et al. 2012, 2018). Despite the known high virulence of RAPD-group B (Jussila et al. 2013; Viljamaa-Dirks et al. 2016; Jussila et al. 2017), *A. leptodactylus* seems to be able to suppress an infection by this group (Maguire et al. 2016; Kokko et al. 2018). This may indicate an adaptation of *A. astaci* and indigenous European crayfish. The increased biotic resistance of a host species towards its pathogens due to the constant contact of the two is known to be characteristic during biological invasions (Faillace and Morin 2016).

Another haplogroup of *A. astaci* was found in the Danube Delta and the invaded part of the Danube River in Serbia. The mitochondrial sequences (Makkonen et al. 2018) assigned the three samples as part of the A-haplogroup. This haplogroup contains the RAPD-groups A and C and the uncultured genotype Up detected once in Czech Republic (Grandjean et al. 2014). In combination with analyses of the chitinase gene (Makkonen et al. 2012a), it was further possible to show similar grouping with RAPD-group C and with microsatellite analysis, one of these samples (DC18) was successfully analyzed, showing an identical allele pattern to the presumed genotype Up. The other samples could not be analyzed with microsatellite analysis, due to low agent levels of the samples. Grandjean et al. (2014) concluded that the genotype Up might originate from *P. leniusculus*, because of its high similarity to the genotypes B and C. However, these genetic groups and genotypes have not been detected on *O. limosus* to date, although identical chitinase and mtDNA sequence grouping was also detected in *A. astaci* strains isolated from German *Orconectes immunis* (Makkonen et al. 2018). *Orconectes limosus* is the only invasive crayfish species recorded in the lower Danube so far (Pârvulescu et al. 2012; 2015) and in Czech Republic it is known to be a carrier of the genetic RAPD-PCR group E (Kozubíková et al. 2011).

The infection prevalence of *O. limosus* in our study was very low (8%) in comparison

to data from the Danube in 2012 with 32% of individuals infected (Pârvulescu et al. 2012). The general range of infection prevalence seems to be high in *O. limosus*, ranging between 0% and 100% (Kozubíková et al. 2009; Maguire et al. 2016). Temporal fluctuations might also be the reason for the low infection prevalence of *O. limosus* in the current study (Matasová et al. 2011). The haplotypes of the infected *O. limosus* samples in this study remained unknown due to the low agent levels found in the samples, which is characteristic of American crayfish species which can prevent the spread of *A. astaci* hyphae in their bodies (Cerenius et al. 2003).

When looking at the current distribution of crayfish species in the lower Danube, it could be hypothesized that *O. limosus* is the source of the *A. astaci* infection, which, however, cannot be proven in this study. If this was the case, *O. limosus* in the Danube would be a carrier of a typical *P. leniusculus* genetic group. Stockings of *O. limosus*, followed by natural diffusion, as well as uncontrolled spreading by anglers and water body owners, helped the species to extend its range and invade several European countries (Souty-Grosset et al. 2006; Kouba et al. 2014). During this human-mediated spread, the species might have been in contact with *P. leniusculus,* which could have provided an opportunity for the A-haplogroup and B-haplogroup to infect *O. limosus*. The species was introduced into the Hungarian Danube catchments in the late 1950s. From Hungary, *O. limosus* spread along the Danube (Maguire and Klobučar 2003; Pavlović et al. 2006) and reached the Romanian Danube in 2008 (Pârvulescu et al. 2009) where it coexists with, but slowly displaces, the indigenous *A. leptodactylus* (Pârvulescu et al. 2012; 2015). Another possible explanation might be that the populations of *O. limosus* in the Danube were not infected before they came into contact with *A. astaci* carrying *A. leptodactylus*. Non-infected *O. limosus* populations have been previously found in Europe (Kozubíková et al. 2009; Schrimpf et al. 2013a).

Due to the reasons mentioned above, we can only speculate about how both

haplogroups, A and B, were translocated into the Danube region. Haplotype A might have originated from relic strains (Schrimpf et al. 2012), which might have spread across Europe with the first crayfish plague outbreaks and since then persisted in populations of *A. leptodactylus*. It was estimated that *A. astaci* first occurred in the lower Danube in 1879-1881 (Alderman 1996), ten years earlier than the first recorded *O. limosus* introductions took place in Poland (Souty-Grosset et al. 2006). Several episodes of mass mortalities of indigenous crayfish, which were probably caused by the disease agent *A. astaci*, have been reported in the Romanian literature (Băcescu 1967). Alderman (1996) estimated the spread of crayfish plague into the Dniester River around 1890-1892. The hypothesis that *A. astaci* in the Danube River could actually be a relic would also explain why the pathogen is found ~900 km downstream of the current invasion front of *O. limosus*. An alternative hypothesis is, that the pathogen was transferred to the Danube Delta in a step stone manner (Schrimpf et al. 2012). However, since *A. astaci* is absent from the non-invaded river sectors of the Danube River in Romania and only reoccurs in the Danube Delta, this hypothesis can probably be dismissed. Although *P. leniusculus* populations have not been reported in the Romanian part of the Danube River, the unsuccessful introduction of *P. leniusculus* might also explain the presence of haplogroups B and A in the Danube Delta and the Dniester River.

The continuous expansion of infected *O. limosus* is a threat to indigenous *A. leptodactylus* in Romania. *Orconectes limosus* has not yet been noted in the Danube Delta, but is spreading downstream in the Danube River at a rate of around 15 km per year. It is expected that the species will reach this region in the 2060s (Pârvulescu et al. 2012). Currently, the haplogroup of *O. limosus* in the Danube River is not known. If it carries the RAPD-PCR group E similarly as *O. limosus* in Czech Republic, the simultaneous occurrence of three different haplogroups of *A. astaci* might increase the pressure on indigenous crayfish. Therefore, more samples with higher agent levels are needed to identify the group which *O.*

limosus carries. Ideally, *A. astaci* should be isolated in pure culture.

An infection of the marine decapod species could not be detected in this study. These species are numerous in brackish waters of the Danube Delta (Petrescu et al. 2010; Skolka and Preda 2010) and might thus play a role in the spread of *A. astaci* if the pathogen was able to survive the salinity of the surrounding water. The Black Sea is known to have lower salt concentrations than the mean ocean salinity (Murray et al. 1991). However, many species of the Saprolegniaceae, which also belong to the oomycota, are unable to produce zoospores, even at low salt concentrations (Cerenius and Söderhäll 1985; Rantamäki et al. 1992; Harrison and Jones 1975). As we did not detect any A. *astaci* infections in the marine decapods in this study, we support the hypothesis that *A. astaci* is unable to spread within marine or brackish environments and is thus not able to infect marine decapods.

In conclusion, we tested indigenous crayfish of the Romanian Danube and the Danube Delta positive for *A. astaci* DNA, despite the fact that North American crayfish have not been recorded in the lowest parts of the Danube River. This work also provides evidence of *A. astaci* in an area inhabited by indigenous crayfish populations only, but which is presumed to be historically affected by outbreaks. Since the identified haplogroups of *A. astaci* do not correspond to the one previously detected in invasive *O. limosus*, it is possible that this species is not the original transmitter. In any of the discussed scenarios, it appears that the pathogen and its host might be reaching a natural equilibrium, as the populations of *A. leptodactylus* tested here all coexist with *A. astaci*. This therefore provides seeds hope for the survival of European indigenous crayfish populations.

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7. TABLES AND FIGURES

Table 1 The sampling sites and analyzed individuals for the qPCR detection of *A. astaci* DNA in populations according to the invasion status, NICS = non-indigenous crayfish species. The sites where sequencing and/or genotyping were possible are marked with an asterisk (*).

Sampling site (hydrographical basin)	GPS location (N/E)	NICS invasion status	No. of analyzed individuals	
			A. leptodactylus	*O. limosus*
Stara Palanka (Danube, Serbia)	44°49′37″/21°20′50″	*O. limosus*	2*	0
Dubova (Danube)	44°37′22″/22°16′23″	*O. limosus* since 2012 (Pârvulescu et al., 2012)	24	20
Drobeta-Turnu Severin (Danube)	44°37′17″/22°40′41″	Invasion front of *O. limosus* in 2015 (Pârvulescu et al., 2015)	17	18
Total specimens analyzed in invaded Danube			43	38
Calafat (Danube)	43°59′54″/22°56′02″	No NICS reported so far	8	-
Bechet (Danube)	43°44′48″/23°56′50″	No NICS reported so far	23	-
Giurgiu (Danube)	43°52′04″/25°57′48″	No NICS reported so far	30	-
Total specimens analyzed in non-invaded Danube			61	0
Rosu - Puiu - Lumina lakes (Danube Delta)	45°04′46″/29°31′49″	No NICS reported so far	58*	-
Dubăsari (Dniester River)	47°17′16″/29°08′19″	No NICS reported so far for the whole basin	104*	-

Table 2 The marine decapods species collected from the West coast of the Black Sea, analysed for presence of *A. astaci* DNA using qPCR. GPS location for sampling sites: Constanta (44°14′18″/28°44′27″), Mangalia (43°48′43″/28°37′38″).

Species	Site locality	No. of analyzed individuals
Palaemon elegans, rockpool shrimp	Mangalia	9
Xantho poressa, jaguar round crab	Mangalia, Constanta	8
Pachygrapsus marmoratus, marbled crab	Mangalia, Constanta	8
Liocarcinus holsatus, flying crab	Constanta	8
Pilumnus hirtellus, bristly crab	Constanta	2
Rhithropanopeus harrisii, dwarf crab	Mangalia	1
Pisidia longimana, porcelain crab	Mangalia	1

Table 3 Number of tested specimen of *Astacus leptodactylus* and *Orconectes limosus* from the Danube River, Danube Delta and the River Dniester, respectively, associated agent levels (A0 (0 PFU) and A1 (PFU_{obs} < 5 PFU) are considered uninfected and A2 (5 PFU ≤ PFU_{obs} < 50 PFU), A3 (50 PFU ≤ PFU_{obs} < 10^3 PFU), A4 (10^3 PFU ≤ PFU_{obs} < 10^4 PFU), A5 (10^4 PFU ≤ PFU_{obs} < 10^5 PFU) and A6 (10^5 PFU ≤ PFU_{obs} < 10^6 PFU) infected), the absolute (n infected) as well as the relative (% infected) number of infected specimen and the 95% confidence intervals (95% CI). Highly infected specimens of Schrimpf et al. (2012) were used in this study for sequence analysis and genotyping.

Sampling site	n	Agent level							n infected	% infected	95% CI
		A0	A1	A2	A3	A4	A5	A6			
Danube channel, invaded sector (*A. leptodactylus*)	43	11	23	6	3	0	0	0	9	21	10 – 36
Danube channel non-invaded sector (*A. leptodactylus*)	61	51	10	0	0	0	0	0	0	0	0 – 6
Danube channel (*O. limosus*)	38	19	16	3	0	0	0	0	3	8	2 – 21
Danube Delta (*A. leptodactylus*)	58	25	23	3	6	0	0	1	10	17	9 – 29
Dniester River (*A. leptodactylus*)	104	94	4	2	1	3	0	0	6	6	2 – 12
Sum of all *A. leptodactylus*	266	181	60	11	10	3	0	1	25	9	6 – 14
Sum of all *O. limosus*	38	19	16	3	0	0	0	0	3	8	2 – 21
Sum of all samples	304	200	76	14	10	3	0	1	28	9	6 – 12
Danube Delta (*A. leptodactylus*) (Schrimpf et al. 2012)	37	23	3	5	3	1	1	1	11	30	16 – 47
Marine decapods	37	37	0	0	0	0	0	0	0	0	0 – 10

Table 4 Sequence analysis results for the chitinase, rnnL and rnnS genes. "n" is the number of successfully sequenced samples from each location. "Chitinase" indicates, which RAPD-groups are grouped together by the found sequence. "rnnL/rnnS Haplogroup" indicates which haplogroup was identified for each sample. Haplogroup A contains both RAPD-groups A and C, thus identifying the samples DC18 and ASLSRB45/58 to be RAPD-group C.

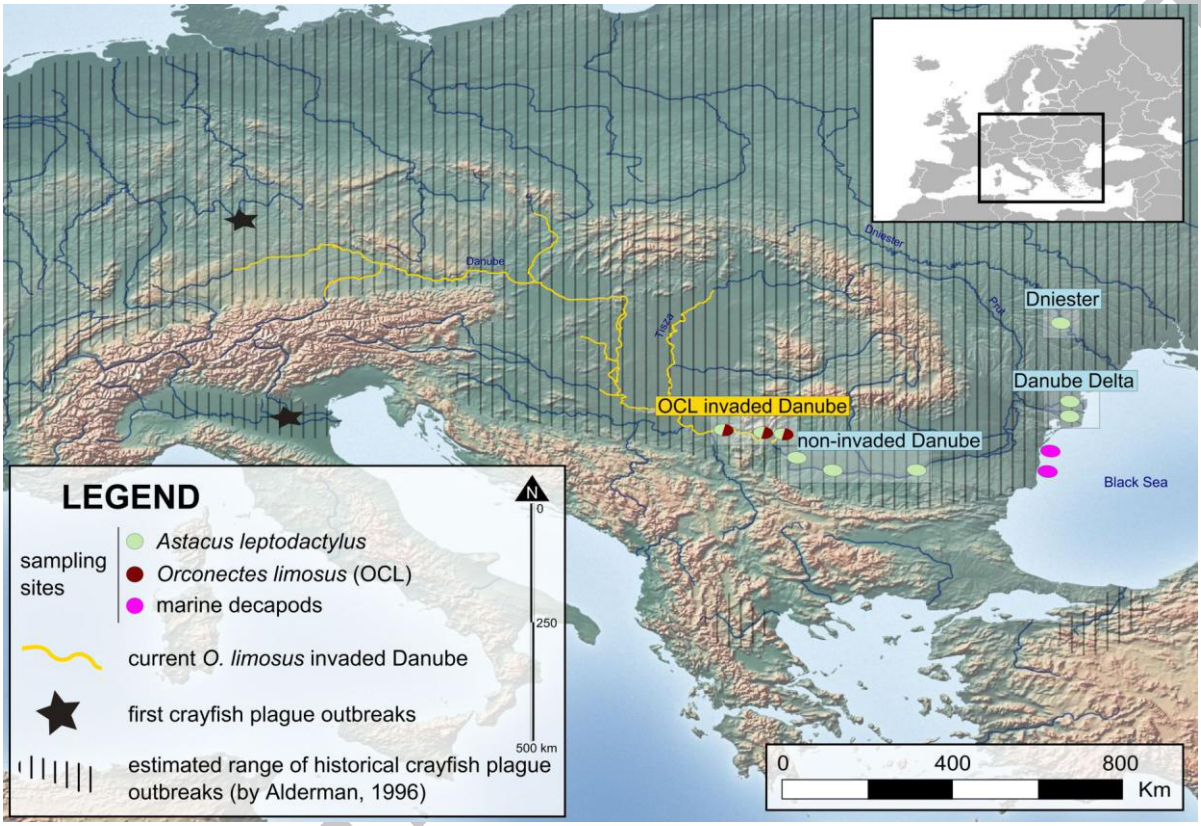
	Sample ID	n	Chitinase	Accession number	rnnL/rnnS Haplogroup	Accession number (rnnL)	Accession number (rnnS)
Danube Delta (2012)	DC18	1	B/C/E*	MF774442	A	MF740803	MF740807
Danube Delta (2015)	DD108	1	B/C/E*	MF774443	B	MF740804	MF740808
Danube River, Serbia (2015)	ASLSRB 35/58	2	B/C/E*	MF774441	A	MF740802/ MF740805	MF740806/ MF740809
Dniester River	LP_AlD 26	1	–		B	MF740801	-

* RAPD-groups B/C/E cannot be distinguished through chitinase sequencing.

FIGURE CAPTIONS

Fig. 1 The map shows crayfish and marine decapods sampling sites across the Lower Danube in the context of the last known invasion status of *O. limosus*. The map also presents the historical coverage area of crayfish plague as estimated by Alderman (1996).

Fig. 1



APPENDIX

Table A Results of the microsatellite analysis using nine co-dominant markers (Grandjean et al. 2014). To compare allele sizes, the fragments from five pure-culture *A. astaci* strains of genotypes A, B, C, D and E, as well as from one mixed DNA sample (Up4) are shown. Sample DC18 was detected in this study and was found on *A. leptodactylus* from the Danube Delta. The allele pattern of sample DC18 is identical to the genotype Up. Allele sizes cannot be directly compared to the results from Grandjean et al. (2014) because they were generated on another sequencer with different color labels. Each reference sample was therefore tested in our own lab for the study at hand.

Code	SSR-A2	SSR-B	SSR-C	SSR-D	SSR-E	SSR-Up	SSR-Up (This study)
Strain	UEF-ATID	UEF-SATR1	Kv1	AP03	Li05 / Li08	Up4	DC18
Aast2	161	145	155	–	151/163	145/151	145/151
Aast4	105	89	89	133	89	89	89
Aast6	160	151	151	151	151/160	151	151
Aast7	207	215	191/215	203	207	205/215	205/215
Aast9	178	164/182	164/168	178	168/180	164	164
Aast10	145	135	135	157	135/145	135/141	135/141
Aast12	–	226/238	226	232	238	226	226
Aast13	195	203	203	195	203	203	203
Aast14	245	247	247	249	247	247	247

Highlights

- *Aphanomyces astaci* detected Danube Delta despite absence of North American crayfish
- Two different haplogroups of *A. astaci* detected
- Found Haplogroups considered untypical for the nearest American crayfish species
- *A. astaci* genetic groups are probably relicts of older crayfish plague outbreaks