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Research Article

Crayfish plague pathogen detected in the Danube Delta – a potential threat to freshwater biodiversity in southeastern Europe

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Abstract

The crayfish plague, caused by the oomycete *Aphanomyces astaci*, is probably the most significant reason for declines in European freshwater crayfish species. One of its hosts, the North American spiny-cheek crayfish *Orconectes limosus*, extends its range in the river Danube and recently reached the territory of Romania. We used highly sensitive *A. astaci*-specific real-time PCR to test if the native narrow-clawed crayfish *Astacus leptodactylus* in the highly protected Danube Delta about 970 km downstream of the current invasion front of American crayfish is a carrier of the crayfish plague. Thirteen out of 40 analysed native *A. leptodactylus* tested positive for the crayfish plague pathogen, infected individuals were found at both sampled localities within the Danube Delta. Therefore *A. astaci* has a much wider range in this river than assumed. The pathogen seems to persist in local populations, as neither crayfish mass mortalities nor alien crayfish species have been reported from the region. *Aphanomyces astaci* may have reached the Delta by long-range passive dispersal of infected hosts or pathogen spores, or by gradually infecting populations of native crayfish in upstream regions of the Danube in a stepping-stone manner. Alternatively, the crayfish plague may have persisted in the Danube Delta as chronic infection from an old plague wave in the 19th century. In any case, the presence of this pathogen in the lower Danube may become a threat to conservation of European crayfish and to freshwater biodiversity in many regions of southeastern Europe, at present considered "crayfish plague-free".

Key words: *Aphanomyces astaci; Astacus leptodactylus;* Black Sea; crayfish plague; Danube Delta; freshwater biodiversity; invasive species

Introduction

The distribution of freshwater organisms is affected by varying climatic cycles and topographic features (e.g. changes in river flow) and in the last centuries to a high degree by direct and indirect human impacts (e.g. by species translocations, habitat alterations, and anthropogenic pollution). For several decades, the distribution and abundance of native European crayfish species has been strongly affected by the crayfish plague (Holdich 2002). The oomycete *Aphanomyces astaci* Schikora, the causative agent of this disease, had been most probably introduced from North America to Europe in the late 1850s together with some of its original hosts, North American freshwater crayfish species (Alderman 1996). This aggressive pathogen is listed among the world´s 100 worst alien species (Lowe 2004) because of its devastating effects resulting in mass mortalities of whole populations of European freshwater crayfish. Since its introduction, the crayfish plague has destroyed many European crayfish populations and caused substantial losses to wild crayfish stocks as well as to valuable fisheries (Alderman 1996). Three of its original host species in Europe extended their range within the continent enormously by active migration as well as by human-mediated dispersal. The widespread presence of American crayfish populations that serve as reservoirs of the pathogen (e.g. Kozubíková et al. 2009) makes it a continuous threat to local crayfish (Holdich et al. 2009). Up to now, it has been believed that all native crayfish from Europe are highly susceptible to crayfish plague, and that infection by the plague pathogen generally leads

to their death. However, a few recent studies reported that native crayfish populations may persist for several years or even decades with certain levels of infection by *A. astaci* (Jussila et al. 2011; Kokko et al. 2012; Svoboda et al. 2012). Despite these exceptional cases, crayfish plague poses a high risk to waters not yet affected by the disease, especially in eastern European countries where it is less widespread (Holdich et al. 2009).

In the lower Danube basin, there had been no reports of outbreaks of this disease for decades, although the whole river had been substantially affected by crayfish plague in the late 19th century (Alderman 1996). However, as in other parts of Europe, American crayfish had not been reported from the Danube basin during that first huge infection wave but colonised it only much later. The spiny-cheek crayfish (*Orconectes limosus* Rafinesque, 1817) was recorded for the first time in the river in 1985 in Hungary (Puky and Schád 2006). Since then it has spread along its course and reached Romania by 2008 (Pârvulescu et al. 2009). There it coexists and slowly displaces populations of native narrow-clawed crayfish (*Astacus leptodactylus* Eschscholtz, 1832) (Pârvulescu et al. 2012). Spiny-cheek crayfish populations in the Danube were repeatedly shown to host *A. astaci* (Kozubíková et al. 2010; Pârvulescu et al. 2012). Interestingly, the presence of this pathogen was also confirmed by molecular methods in healthy-looking individuals of narrow-clawed crayfish ~70 km downstream of the presumed invasion front of spiny-cheek crayfish in the Danube (Pârvulescu et al. 2012) in August 2011. This sampling site is located ~900 km upstream of the river delta.

The downstream colonization rate of the Danube by the spiny-cheek crayfish was estimated to about 13-16 $km \cdot yr^{-1}$ (Puky and Schád 2006; Pârvulescu et al. 2012). At this rate, this invasive species would reach the Danube Delta no sooner than in the 2070s. However, the presence of *A. astaci* in healthy-looking narrow-clawed crayfish (Pârvulescu et al. 2012) suggested that the crayfish plague pathogen may be steadily spreading ahead of the invasion front of American crayfish. The knowledge of the geographic distribution of the crayfish plague pathogen is important in order to prioritize conservation management of the most endangered populations. Presence and absence data of *A. astaci* is, for example, important for the concept of "ark sites", a common component of modern management plans for endangered crayfish in Europe (Kozák et al. 2011). An ark site is an isolated refuge site where native crayfish species are not at the risk from adverse factors, including colonization by invasive crayfish species and *A. astaci* (Peay 2009). Individuals from non-infected populations that are in danger of getting infected by crayfish plague in the near future can be translocated to ark-sites to save them from dying and to conserve the intraspecific diversity. Translocation from an infected population, on the other hand, holds the threat of further spreading the agent of the crayfish plague.

The Danube Delta is included in the UNESCO List of World Natural Heritage Sites (UNESCO 2012) because of its outstanding biodiversity. An infection of native crayfish with *A. astaci* in this highly protected area may have dramatic consequences. The elimination of native crayfish may lead to a strong cascading effect in the whole food web with unpredictable implications for other freshwater organism. To evaluate if the concept of ark sites can be applied to crayfish from the Danube Delta and to further provide a basis for conservation management measures, we tested native narrow-clawed crayfish from the Danube Delta for possible infection by *A. astaci*, using a highly specific and sensitive molecular method to detect pathogen DNA.

Methods

In May 2011, we captured freshwater crayfish at two different places within the Danube Delta, in the Chilia main channel and in the Merhei Lake (Figure 1). Water exchange between the channel and the lake, which are connected by 12 kilometres of a narrow canal, is very low. Five days were spent along a 5-km stretch of the Chilia Channel (between 45.31N, 29.67E and 45.27N, 29.68E) by capturing crayfish into traps. Due to a difficult access, only one daylight capture was possible in the Merhei Lake (45.32N, 29.45E), by using fish-baited nets. Additionally, we checked a local fish market and catches of five local fishermen around the Danube Delta for any non-native crayfish species that might be available for sale.

To avoid any potential cross-contamination by the pathogen, we used new traps and a toolbox without any previous contact with crayfish. As whole specimens of narrow-clawed crayfish cannot be collected in this protected area, only two parts of the uropods and, if present, one melanised walking leg, were sampled from each

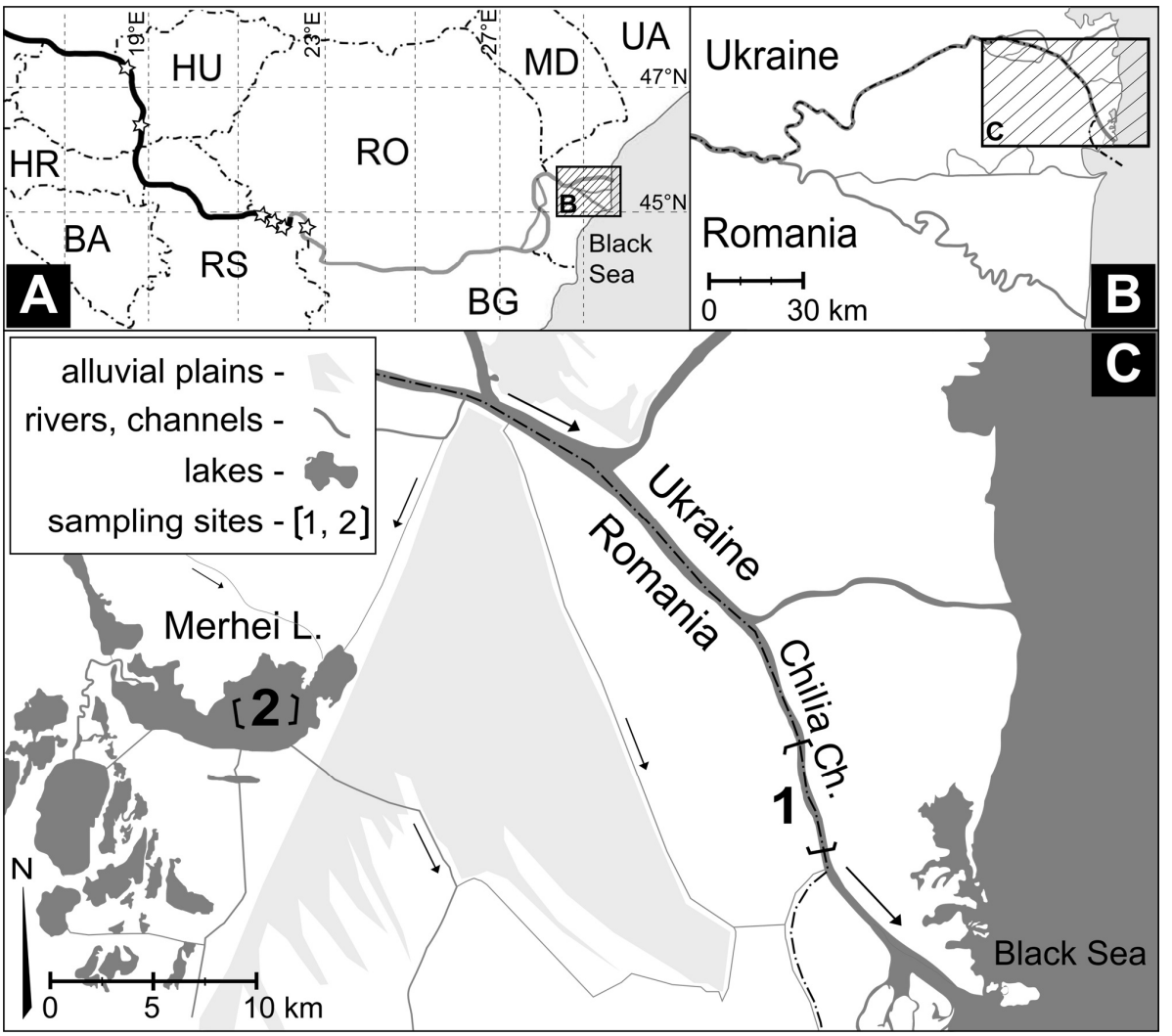

Figure 1. **A**: Overview of the distribution of *Orconectes limosus* (dark line) and the sites with confirmed presence of the crayfish plague pathogen (stars) in the Danube; **B**: Overview of the Danube Delta; **C**: Sampling sites in the Danube Delta (1- Chilia Channel, 2- Merhei Lake). The arrows indicate the water flow direction.

crayfish and stored in 96% ethanol. We extracted DNA from the crayfish tissues as described in Vrålstad et al. (2009). For detection of *A. astaci* DNA, we used the quantitative TaqMan® minor groove binder (MGB) real-time PCR (Vrålstad et al. 2009), the most sensitive and specific detection assay available at present (Tuffs and Oidtmann 2011). Real-time PCR reaction was performed on a Mastercycler® ep realplex S (Eppendorf) using the TaqMan® Environmental Master Mix to avoid PCR inhibition (Strand et al. 2011). We increased the annealing temperature to 62°C and decreased the annealing time to 15 seconds to further exclude possible false positive results (T. Vrålstad, unpublished data; see also Kozubíková et al. 2011b).

To increase credibility of results, the sample set was subdivided into three subsets and different people tested each subset on a different day, with similar results. Apart from using negative controls (i.e., samples containing no DNA), we ruled out potential laboratory contamination by analysing one sample subset together with presumably non-infected spiny-

Table 1. Real-time PCR detection of *Aphanomyces astaci* in investigated specimens of *Astacus leptodactylus* from the Danube Delta.

Sampling site	No. of analysed crayfish	*A. astaci*-positive	Agent level[a]						
			A_0	A_1	A_2	A_3	A_4	A_5	A_6
Chilia Channel	37	11 (30%)	23	3	5	3	1	1	1
Merhei Lake	3	2 (67%)		1	2				
Total	40	13 (32%)							

[a]Relative levels of infection (expressed as agent level after Vrålstad et al. 2009) are given. Individuals with agent levels A_0 (no detection) and A_1 (very low real-time PCR signal, corresponding to less than 5 PFU_{obs}, i.e., PCR forming units observed in the reaction) are conservatively considered uninfected. Individuals with agent levels A_2 and over (A_2: $5 \leq PFU_{obs} < 50$, A_3: $50 \leq PFU_{obs} < 10^3$, A_4: $10^3 \leq PFU_{obs} < 10^4$, A_5: $10^4 \leq PFU_{obs} < 10^5$; A_6: $10^5 \leq PFU_{obs} < 10^6$) are considered infected by *A. astaci*.

cheek crayfish from German populations coexisting with susceptible crayfish. All negative controls, including DNA isolates from these American crayfish, tested negative.

We carried out data analysis using the software Real Plex 2.2 (Eppendorf). The relative level of infection by the pathogen depends on the strength of the real-time PCR signal and corresponding amounts of PCR-forming units in the reaction, and is expressed as semi-quantitative agent levels (according to Vrålstad et al. 2009; see also Table 1).

Results

Altogether 40 individuals of narrow-clawed crayfish were collected in the Danube Delta, 37 from the Chilia Channel and three from Merhei Lake. No other crayfish species were observed, and all investigated crayfish specimens (approximately 600 individuals) from commercial fish captures were identified as the native narrow-clawed crayfish.

Presence of *Aphanomyces astaci* was confirmed at both sampling sites. Overall, DNA of the pathogen was detected by the real-time PCR in 32% of analysed narrow-clawed crayfish specimens (Table 1): in 11 out of 37 individuals captured from the Chilia Channel, and in two out of three specimens from the Merhei Lake. Most samples contained low levels of pathogen DNA ($7 \times$ agent level A_2, $3 \times A_3$); however, three individuals from the Chilia Channel were highly infected (agent level A_4 or more). Melanised areas were observed in the walking legs and in the telson, uropods or abdominal cuticle of four individuals from the Chilia Channel, all of which were infected by *A. astaci*.

Discussion

We have demonstrated that a substantial proportion of narrow-clawed crayfish populations in at least some parts of the Danube Delta were infected by *Aphanomyces astaci*, although no mass mortalities of crayfish have been reported from this region for several decades. The observed levels of infection might still be underestimated, as various body parts in which *A. astaci* may be present could not be analysed due to species conservation legislation. However, crayfish plague detection rate in signal crayfish (*Pacifastacus leniusculus* Dana, 1852) was almost as high for uropods (the tissue of choice in this study) alone as for multiple body parts (Vrålstad et al. 2011). Melanised spots that were observed in four infected narrow-clawed crayfish could have been a visible indication of infection (as considered e.g. by Nylund and Westman 2000) but such spots also appear due to immune reaction to other pathogens and after a mechanical injury (Schulz et al. 2006). In a Turkish population of *A. leptodactylus* in which *A. astaci* seems to persist for over two decades, melanisation was observed on crayfish infected by the pathogen as well as on those in which real-time PCR assay did not confirm its presence (Svoboda et al. 2012). On the other hand, an absence of melanised spots is not an indication of pathogen-free crayfish. We did not notice these signs in the other nine positive as well as in negative tested narrow-clawed crayfish. These symptoms should be thus interpreted with care.

The capture success in the Merhei Lake, from which we obtained only three individuals, was low. Although no unusually high crayfish mortalities have been reported from the Danube Delta recently, we cannot rule out the possibility

that a population decline already took place in this lake. However, the catching was constrained by the fact that we could only spend one day at this lake and the access to the water edge was limited.

Recent dispersal of the pathogen

As no non-native crayfish species have ever been reported from the Danube Delta, nor were they captured in the investigated areas or observed on the markets, it is unclear how *A. astaci* has reached the region. One plausible possibility is that the infection originates from American crayfish in the upstream regions of the Danube, and has spread downstream much earlier and faster than expected by Pârvulescu et al. (2012). There are several potential processes that may have ensured long-range pathogen dispersal: (i) The plague pathogen could have been infecting native narrow-clawed crayfish that are widely distributed in the lower Danube in a stepping-stone manner, by short-range downstream dispersal of zoospores between susceptible animals. Since the survival of spores in freshwater is at least seven days at 14°C (Unestam 1969), the spores, if released, may be carried in running water for around 150 kilometres considering a current velocity of 0.5 $m \cdot sec^{-1}$. (ii) Passive transport of infected live or dead crayfish (or even their exuviae) may have contributed to the pathogen dispersal since *A. astaci* remains viable for at least five days in a crayfish cadaver (Oidtmann et al. 2002). (iii) Other animals, particularly fish feeding on infected crayfish, may transport the pathogen from infected to healthy populations (Oidtmann et al 2002). (iv) Boats that frequently pass through the Danube could transport non-native crayfish or pathogen spores (e.g. in the ballast water) for long distances. We also cannot rule out an undetected expansion of spiny-cheek crayfish to some lower reaches of the Danube.

Relic from an old infection wave?

An alternative explanation for the origin of the present infection in the Danube Delta is that the crayfish plague pathogen could be a relic from the original infection wave that caused mass mortalities in crayfish along the Danube in the 19th century (Alderman 1996). Since then, the pathogen might have persisted, possibly as chronic infection, in local narrow-clawed crayfish populations for more than a century. This would contradict the general assumption that the pathogen is lethal to this native European crayfish species. However, there is some evidence that long-term coexistence of narrow-clawed crayfish and *A. astaci* may be possible. In particular, it has been repeatedly reported that *A. astaci* persists in some Turkish lakes inhabited by narrow-clawed crayfish since the mid-1980s (e.g., Harlioğlu 2008), and this has been supported by the recent molecular detection of *A. astaci* (Svoboda et al. 2012; Kokko et al. 2012). Similarly, coexistence of presumably even more sensitive noble crayfish *Astacus astacus* (Linnaeus, 1758) with *A. astaci* was recently reported from two Finnish lakes in which the crayfish plague agent had probably been present for several years without a proven presence of alien crayfish (Jussila et al. 2011; Viljamaa-Dirks et al. 2011).

Several mechanisms that may promote coexistence between native European crayfish and the crayfish plague pathogen have been discussed but so far they remain unclear (Jussila et al. 2011; Viljamaa-Dirks et al. 2011; Svoboda et al. 2012). It is assumed that some native crayfish exhibit certain levels of tolerance to the infection, possibly strengthened by a strong selection pressure due to the initial crayfish plague outbreaks. Furthermore, it is possible that the pathogen has also adapted to the new hosts and lowered its virulence over the years since it is evolutionary disadvantageous from the pathogen´s perspective when the host population dies out. In order to resolve this question, laboratory experiments with susceptible crayfish exposed to *A. astaci* should be designed and potential differences in crayfish mortality rates recorded. The settings should vary with respect to (i) the origin of *A. astaci* (regions where either latent infections of susceptible crayfish or mass mortalities are observed), (ii) the origin of crayfish hosts (populations coexisting with the pathogen, such as those in the Danube Delta or Turkey, vs. other regions), (iii) *A. astaci* spore concentration (which likely varies with different host densities).

It seems that different American crayfish species carry distinct pathogen strains (Huang et al. 1994; Kozubíková et al. 2011a), which might differ in their virulence to European crayfish. For instance, signal crayfish host other *A. astaci* strains than those involved in the first crayfish plague infection wave or those recently isolated from spiny-cheek crayfish (Kozubíková et al. 2011a). The signal crayfish currently extends its

range to the Danube tributaries in Croatia (Hudina et al. 2009) and may reach the lower Danube in the future, possibly introducing a different *A. astaci* strain that might be more virulent to narrow-clawed crayfish. It appears that signal crayfish rapidly displaced some native crayfish populations in Croatia (Hudina et al. 2009), probably due to transmission of *A. astaci*. Although narrow-clawed crayfish in the Danube Delta seems to persist at present in the presence of *A. astaci*, this might change in case of infections by a more virulent strain of this pathogen.

Threat to freshwater biodiversity

Despite occasional reports on coexistence of populations of European crayfish with *A. astaci*, we have to assume that native crayfish species are threatened by further dispersal of the crayfish plague agent, and local stakeholders should react accordingly. Besides narrow-clawed crayfish, this applies to native noble crayfish and stone crayfish (*Austropotamobius torrentium* Schrank, 1803), distributed in the drainage area of the lower Danube (Holdich et al. 2009) and to the mesohaline thick-clawed crayfish (*Astacus pachypus* Rathke, 1837), which occurs only in small parts of the Black and Caspian seas (Holdich et al. 2006). The Southern Balkans, a glacial refuge for many freshwater species (Hewitt 1999), harbours high genetic diversity within European crayfish (Trontelj et al. 2005; Schrimpf et al. 2011).

It is possible that we have detected the infection in the Danube Delta in an early stage and that mass mortalities will follow, as described by Alderman (1996) for the 1890s. Especially when spores are dispersed in low densities and water temperature is low, mortalities may not be apparent for a long time (Alderman et al. 1987). Therefore, crayfish populations in the Danube Delta might be hit by crayfish plague outbreaks associated with high crayfish mortalities when the water temperature increases in the summer. European crayfish, as the largest freshwater invertebrates and due to their trophic activities as omnivores, play a key role in many freshwater ecosystems (Nyström 1999), and their loss may have drastic impacts on local biodiversity. This is relevant for all regions threatened by crayfish plague but might be particularly important in the wetlands of the Danube Delta.

Conservation implication

Since funding for conservation is often limited, a precise knowledge of the situation and accurate prediction of sites most at risk of becoming invaded by non-native species is fundamental to create an effective management plan (Keller et al. 2008). We presume *A. astaci* is probably present along the whole Danube main channel, therefore translocations of freshwater crayfish as well as conservation actions on crayfish (e.g. the concept of ark sites) in the Danube have to be considered carefully. On the other hand, the side channels of the Danube could be prioritized for management actions. Native crayfish populations in the side channels that are threatened by a further dispersal of *A. astaci* and are negatively tested for an infection could be translocated to ark sites to avoid further loss of within-species diversity. Furthermore, since many invasive species, including North American crayfish and the crayfish plague pathogen carried by them, are difficult or impossible to eradicate, the best option for limiting total impacts is often to restrict spread (Keller et al. 2008). Therefore, local stakeholders, communities and fishermen need to get informed about the high risk of spreading invasive crayfish or even native crayfish infected with *A. astaci*.

An important question is whether *A. astaci* will be able to disperse from the Danube through the brackish Black Sea (with an average salinity of 18 psu) into other river basins. It remains open whether it could survive in hosts that tolerate salinities up to 21 psu (e.g. narrow-clawed crayfish, signal crayfish and spiny-cheek crayfish; Jażdżewski et al. 2005). Furthermore, the dispersal of the pathogen might be possible by ship traffic. To avoid spreading of *A. astaci* through plague-free eastern European countries by cargo ships, ballast water treatment systems should be strictly implemented.

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