# DNA-based methods for freshwater biodiversity conservation

# -

# Phylogeographic analysis of noble crayfish (*Astacus astacus*) and new insights into the distribution of crayfish plague

DISSERTATION

zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften

Fachbereich 7: Natur- und Umweltwissenschaften
der Universität Koblenz-Landau
Campus Landau
vorgelegt

am 16. Januar 2013

von Anne Schrimpf
geboren am 21. September 1984 in Frankfurt am Main

Referent: **Prof. Dr. Ralf Schulz**
Koreferent: **Prof. Dr. Klaus Schwenk**

*- This thesis is dedicated to my grandparents -*

# CONTENT

## FIGURES



## TABLES

# ABSTRACT

Worldwide one third to one half of the freshwater crayfish species are threatened with population decline or extinction. Besides habitat deterioration, pollution, and other man-made environmental changes, invasive species and pathogens are major threats to the survival of European crayfish species. Freshwater crayfish are the largest freshwater invertebrates and strongly influence the structure of food webs. The disappearance of crayfish from a water body may change the food web and could have dramatic consequences for an ecosystem.

One goal in modern species conservation strategies is the conservation of genetic diversity, since genetic diversity is an advantage for the long-term survival of a species. The main aim of my thesis was to reveal the genetic structure and to identify genetic hotspots of the endangered noble crayfish (*Astacus astacus*) throughout Europe (part 1 of my thesis). Since the most significant threat to biodiversity of European crayfish species is the crayfish plague pathogen *Aphanomyces astaci* I studied new aspects in the distribution of *A. astaci* (part two of my thesis). The results serve as a basis for future conservation programs for freshwater crayfish.

In the first part of my thesis I conducted a phylogeographic analysis of noble crayfish using mitochondrial DNA and nuclear microsatellite data. With these methods I aimed to identify its genetic hotspots and to reconstruct the recolonization history of central Europe by this species. I detected high genetic diversities in southestern Europe indicating that noble crayfish outlasted the cold climate phases during the Pleistocene in this region (***Appendix 1***). Because of the high genetic diversity found there, southeastern Europe is of particular importance for the conservation of noble crayfish. The mitochondrial DNA analysis points to a bifurcated colonization process from the eastern Black Sea basin to a) the North Sea and to b) the Baltic Sea basin (***Appendix 2***). A second independent refugium that was localized on the Western Balkans did not contribute to the colonization of central Europe. Furthermore, I found that the

natural genetic structure is dissolved, probably due to the high human impact on the distribution of noble crayfish (e.g. artificial translocation).

In the second part of this thesis using real-time PCR I identified calico crayfish (*Orconectes immunis*) as the fourth North American crayfish species to be carrier of the agent of the crayfish plague (***Appendix 3***). Furthermore I detected the crayfish plague pathogen in American spiny-cheek crayfish (*Orconectes limosus*) and native narrow-clawed crayfish (*Astacus leptodactylus*) in the lower Danube in Romania (***Appendix 4***). The distribution of infected spiny-cheek crayfish poses a threat to the native biodiversity in southeastern Europe and shows the high invasion potential of this crayfish species. Moreover, I found that even the native narrow-clawed crayfish in the Danube Delta, about 970 km downstream of the current invasion front of American crayfish, is a carrier of *A. astaci* (***Appendix 5***). This finding is of high importance, as the native species do not seem to suffer from the infection. In ***Appendix 6*** I elucidate demonstrate that the absence of the crayfish plague agent is the most likely explanation for the coexistence of populations of European and American crayfish in central Europe. In my thesis I show that the common assumption that all North American crayfish are carrier of *A. astaci* and that all native crayfish species die when infected with *A. astaci* does not hold true.

The studies presented in my thesis reveal new aspects that are crucial for native crayfish conservation:

1) The genetic diversity of noble crayfish is highest in southeastern Europe where noble crayfish outlasted the last glacial maximum in at least two different refugia.

2) Not all American crayfish populations are carrier of *A. astaci* and

3) not all Europen crayish populations die shortly after being infected with the crayfish plague pathogen.

To conserve native crayfish species and their (genetic) diversity in the long term, further introductions of American crayfish into European waters must be avoided. However, the introduction will only decrease if the commercial trade with non-indigenous crayfish species is prohibited.

# ZUSAMMENFASSUNG

Weltweit sind ein Drittel bis die Hälfte der Flusskrebsarten von Populationsrückgang oder Aussterben bedroht. Neben einer Verschlechterung der Habitate, Umweltverschmutzung und anderen vom Menschen verursachten Umweltveränderungen stellen eingeschleppte exotische Arten und Krankheitserreger eine große Bedrohung für das Überleben europäischer Flusskrebsarten dar. Flusskrebse sind die größten Wirbellosen in limnischen Systemen und haben einen entsprechend großen Einfluss auf die Struktur der Nahrungsnetze. Das Verschwinden von Flusskrebsen aus einem Gewässer kann Nahrungsnetze verändern und somit dramatische Konsequenzen für ein Ökosystem zur Folge haben.

Ein Ziel im modernen Artenschutz ist die Erhaltung der genetischen Vielfalt. Eine hohe genetische Vielfalt ist für das langfristige Überleben einer Art von Vorteil. Das Hauptziel meiner Arbeit war es, die genetische Struktur des gefährdeten Edelkrebses (*Astacus astacus*) in seinem europäischen Verbreitungsgebiet zu untersuchen und die besonders schützenswerten genetische 'Hotspots' zu identifizieren (Teil 1 der Dissertation). Die größte Bedrohung für die Diversität europäischer Flusskrebsarten stellt der Krebspesterreger *Aphanomyces astaci* dar. Daher muss die Verbreitung des Krankheitserregers bei Schutzprogrammen beachtet werden. Im zweiten Teil der Dissertation untersuchte ich neue Aspekte der Verbreitung von *A. astaci.* Die Ergebnisse dienen als Grundlage für zukünftige Artenschutzprogramme für Flusskrebse.

Im ersten Teil dieser Arbeit führte ich eine phylogeographische Analyse der Edelkrebse durch, um genetische 'Hotspots' zu identifizieren und die nacheiszeitliche Wiederbesiedlung Zentraleuropas durch diese Art zu rekonstruieren. Mit mitochondrialer DNA und nuklearen Mikrosatelliten-Markern ermittelte ich eine hohe genetische Vielfalt in Südosteuropa, die darauf hinweist, dass der Edelkrebs die kalten Klimaphasen des Pleistozäns in diesem Gebiet überdauerte (***Appendix 1***). Wegen der hohen genetischen Vielfalt ist Südosteuropa von besonderer Bedeutung für den Schutz des Edelkrebses. Die

mitochondriale DNA-Analyse deutet auf eine gegabelte Kolonisierung vom unteren Donaueinzugsgebiet in a) das Einzugsgebiet der Nordsee und b) das Einzugsgebiet der Ostsee hin (Kapitel 2). Ein zweites, unabhängiges Refugium, welches im westlichen Balkan lokalisiert wurde, hat vermutlich nicht zur Besiedlung Mitteleuropas beigetragen. Außerdem stellte ich fest, dass die natürliche genetische Struktur teilweise überlagert ist, wahrscheinlich aufgrund des hohen menschlichen Einflusses auf die Verbreitung des Edelkrebses (bspw. künstliche Translokation).

Im zweite Teil dieser Arbeit konnte ich mittels real-time-PCR ermitteln, dass neben den bekannten drei nordamerikanischen Flusskrebsarten auch Kalikokrebse (*Orconectes immunis*) Träger des Krebspesterregers sind (Kapitel 3). Des Weiteren habe ich den Krebspesterrreger in der unteren Donau in Rumänien an amerikanischen Kamberkrebsen (*Orconectes limosus*) und europäischen Galizierkrebsen (*Astacus leptodactylus*) nachweisen können (Kapitel 4). Die Ausbreitung der infizierten Kamberkrebse bis in die untere Donau stellt eine große Bedrohung für die Artenvielfalt in Südosteuropa dar und zeigt das hohe Invasionspotential der Kamberkrebse. Darüber hinaus stellte ich fest, dass auch einheimische Galizierkrebse im Donaudelta, etwa 970 km hinter der aktuellen Invasionsfront des Kamberkrebses, Träger von *A. astaci* sind (Kapitel 5). Diese Erkenntnis ist von besonderer Bedeutung, da die einheimischen Arten offenbar nicht an der Infektion leiden. Die Untersuchung koexistierender Populationen europäischer und amerikanischer Flusskrebse ergab, dass die Abwesenheit des Krebspesterregers in diesen Populationen die wahrscheinlichste Erklärung für die erfolgreiche Koexistenz in den untersuchten Gewässern in Mitteleuropa ist (Kapitel 6).

Die Ergebnisse meiner Dissertation zeigen neue Aspekte, die von hoher Relevanz für den Schutz und Erhalt einheimischer Flusskrebsarten und deren genetischer Vielfalt sind:

1) Die genetische Diversität des Edelkrebses ist in Südosteuropa am höchsten. Dort überdauerten Edelkrebse die letzte Eiszeit in mindestens zwei unabhängigen Refugien.

2) Nicht alle amerikanischen Flusskrebspopulationen sind Träger der Krebspest und

3) nicht alle europäischen Flusskrebspopulationen sterben innerhalb kurzer Zeit an einer Infizierung mit dem Krebspesterreger.

Um einheimische Flusskrebse und deren (genetische) Vielfalt langfristig zu erhalten, dürfen keine weiteren amerikanischen Flusskrebse in der Natur ausgesetzt werden. Das unbefugte Aussetzen wird jedoch erst zurückgehen, wenn der Handel mit exotischen Flusskrebsen verboten wird.

# *ABBEREVIATIONS*

CBD = Convention on Biological Diversity

COI = cytochrome oxidase subunit I

DNA = deoxyribonucleic acid

ESU = evolutionary significant unit

$H_D$ = haplotype diversity

IUCN = International Union for Conservation of Nature

MGB = minor groove binder

mtDNA = mitochondrial DNA

N = number of samples

$N_+$ = number of positive samples

NICS non-indigenous crayfish species

PCR = polymerase-chain reaction

qPCR = real-time polymerase-chain reaction

rRNA = ribosomal ribonucleic acid

YBP = years before present

# GENERAL INTRODUCTION

## Conservation of biological diversity

In the Convention on Biological Diversity (CBD) negotiated at the United Nations Conference on Environment and Development in 1992, the conservation of biological diversity is highlighted as one of the three main goals (SCBD 1992). Biological diversity is understood as the variability among living organisms from all sources including diversity within and between species and also of ecosystems (CBD, Article 2). To maintain the genetic diversity within one species, conservation genetics aim to protect genetic variability within and between populations. The guidelines for the protection of threatened species recommend the identification of evolutionary significant units (ESU, Ryder 1986). An ESU can be defined as a population or group of populations that is sufficiently differentiated from all the other members of this species and requires an individual management or conservation strategy (Moritz 1994). An ESU may contain edemic genetic variation. In restocking programs, it is therefore recommended to use donor populations from the same ESU whenever possible to conserve the local specificity and maintain the maximum within-species diversity. Species with greater genetic diversity are more likely to be able to evolve in response to a changing environment than those with less diversity (Malcolm *et al.,* 2007).

One of the most significant threats of biodiversity is the introduction of invasive species (McGeoch *et al.,* 2010) and diseases and parasites associated with them. Invasive species are often lacking natural predators and are competitors with the native species (Schulz *et al.,* 2006, Westman and Savolainen 2001). Although introduced species may increase the local biodiversity, they at the same time lead to a global homogenization and therefore to a loss of worldwide biodiversity (McNeely *et al.,* 2001). Compared to terrestrial ecosystems, aquatic ecosystems are particularly subject to invasive species (Dudgeon *et al.,* 2006). The connectivity of river systems facilitates the dispersal of invasive species over large distances. In addition, today artificial channels between

different rivers additionally increase the long-distance dispersal of freshwater species (Bij de Vaate *et al*., 2002).

Otherwise, this connectivity between river systems also aided in the recolonization of the European continent by freshwater species after cold climate phases in the Pleistocene. When climate conditions were unfavorable, e.g. cold, the ranges of most European species contracted to southern Europe (Taberlet *et al.,* 1998, Durand *et al.,* 1999; Theissinger *et al.,* 2012). Range contractions were followed by range expansions when the climate warmed again. The range expansion of freshwater species was most strongly influenced by the changes in landscapes through glaciers and melting water (e.g. Hänfling *et al*., 2009, Vonlanthen *et al*., 2007). This rapid northward expansion led to a reduction in genetic diversity of northern populations. In contrast, the varied topography of southern refugia allowed populations to diverge through several ice ages (Hewitt 1999). Thus, in species with a refugial area in southern Europe a genetic divergence between the ancestral populations in the South and the newly founded populations in the North of Europe can be detected as/in a decrease in genetic diversity (Reiland *et al.,* 2002). For conservation management of a species it is important to reveal the two following aspects: 1) Where were the Pleistocene glacial refugia located? Because of their high genetic diversity, their identification is fundamental for conservation prioritization. 2) Where are possible ESUs? The reconstruction of the phylogeographical distribution aids in defining ESUs, which then serve as a basis for conservation programs. For freshwater species that often cannot migrate over terrestrial habitats, river catchments (e.g. Rhine river catchment, Danube river catchment) and sea basins (North Sea basin, Baltic Sea basin) play a significant role in subpopulational differentiation.

## *The freshwater crayfish*

### General

Freshwater crayfish are the largest freshwater invertebrates (Holdich 2002a). They occupy a variety of streams, rivers and lakes and prefer habitats with an access to shelter. Some crayfish species can have a physical impact on their environment due to their burrowing activity (Correia and Ferreira 1995). Crayfish are often key organism in food webs and have a high influence on its structure due to their omnivore behaviour (Nyström 1999). Besides on invertebrates, they feed on vertebrates like amphibians (i.e. on their eggs and larvae (Axelsson *et al*., 1997)) and on fish (Guan and Wiles 2002) and therefore have direct and indirect influence on the species composition of their respective ecosystems. Freshwater crayfish (Astacida) belong to the monophyletic Reptantia within the Decapoda (Scholz and Richter 1995) and are taxonomically distributed among three families, two in the northern Hemisphere (Astacidae and Cambaridae) and one in the southern Hemisphere (Parastacidae), specifically in Madagascar, southern South America and Australasia (Holdich 2002a). The Cambaridae are naturally found in the Eastern United States and in East Asia, whereas the Astacidae are distributed in the Western United States and in Europe. The diversity of crayfish in Europe is relatively low compared to that in Australia and North America. Freshwater crayfish have two centres of diversity, one in the southeastern USA and one in Victoria, Australia (Crandall *et al.,* 2006). Today more than 460 crayfish species from North America are known (Crandall and Buhay 2011). In Europe, depending on the taxonomy, five or six species from two genera can be distinguished. Narrow-clawed crayfish (*Astacus leptodactylus* Eschscholz 1823), thick-clawed crayfish (*Astacus pachypus* Rathke 1837) and Noble crayfish (*Astacus astacus* Linnaeus 1758) belong to the genus *Astacus* and have a more northern and eastern distribution compared to stone crayfish (*Austropotamobius torrentium* Schrank 1803) and white-clawed crayfish (*Austropotamobius pallipes* Lereboullet 1858 species complex) which belong to the genus *Austropotamobius*. *A. pallipes* is considered a species complex and its taxonomic status is still under revision (Füreder *et al.,* 2010). Schulz and Grandjean (2005) however argue that based on

genetic data this species complex consists of two separate species, *A. pallipes* and *Austropotamobius italicus*.

Today at least eight non-indigenous crayfish species (NICS) are established in the wild in Europe (Holdich *et al.,* 2009; Chucholl and Pfeiffer 2010). In the literature, a distinction is drawn between the 'Old NICS' and the 'New NICS'. The signal crayfish (*Pacifastacus leniusculus* Dana 1852), the spiny-cheek crayfish (*Orconectes limosus* Rafinesque 1817) and the red swamp crayfish (*Procambarus clarkii* Girard 1852) have been introduced before 1975 whereas the 'New NICS' have been introduced after 1980 (Holdich *et al.,* 2009). In this thesis I have investigated the crayfish plague carrier status of the 'Old NICS' spiny-cheek crayfish (Table 1 and ***Appendices 3-6***) and of the 'New NICS' calico crayfish (*O. immunis* Hagen 1870) (***Appendix 3***).

**Table 1** List of crayfish and crayfish pathogen species included in this thesis.

| Common name | Scientific name | Conservation status | Distribution in Europe |
|---|---|---|---|
| Noble Crayfish | *Astacus astacus* | Vulnerable (IUCN), native | Central and eastern Europe |
| Narrow-clawed crayfish | *Astacus leptodactylus* | Least concern (IUCN), native | Native to the Ponto-Caspian regior today in most European countries |
| Spiny-cheek crayfish | *Orconectes limosus* | Invasive American species, 'Old NIC' | Western, central and eastern Europe |
| Calico crayfish | *Orconectes immunis* | Invasive American species, 'New NIC' | Upper Rhine river catchment in Germany and France |
| Crayfish plague | *Aphanomyces astaci* | Included in: 100 of the worst alien species | Throughout Europe |

**The noble crayfish (*Astacus astacus*)**

While the stone crayfish and white-clawed crayfish species complex has already been subject to intensive molecular studies (e.g. Bertocchi *et al.,* 2008, Gouin *et al.,* 2006, Trontelj *et al.,* 2005), molecular studies of noble crayfish have only been focused on restricted geographical areas (Agerberg 1990, Fevolden *et al.,* 1994, Schulz 2000, Edsman *et al.,* 2002, Schulz *et al.,* 2004, Alaranta *et al.,* 2006). Therefore, a part of my thesis focuses on noble crayfish that is widely distributed across Europe. Its range extends from Russia and the Ukraine in the east, Scandinavia in the North, Greece in the South, and the United Kingdom and France in the west (Edsman *et al.,* 2010).

In the literature, different and partly contradicting taxonomic classifications of the noble crayfish (Figure 1) based on morphological criteria below species level can be found (see Smietana *et al*., 2006 for a review). The most widely accepted taxonomic studies (Karaman 1962, 1963) recognized the three following subspecies: *A. a. colchicus,* which can be found in an isolated area in the Ponto-Caspian region of the upper Rion (Caucasus); *A. a. balcanicus,* which populates the Western Balkans with the drainage system of the river Vardar in Macedonia and Greece and in the Lake Ohrid; the subspecies *A. a. astacus* with three races is distributed in the river catchment of the river Danube and the rivers that open out into the North- and Baltic Sea. Phylogenetic analysis of populations from the distribution area of the subspecies *A. a. astacus* is the subject of ***Appendices 1 and 2***. Karaman (1962) named the three races of *A. a. astacus* according to their distribution: Northrace (*A. a. a. astacus*) in Elbe, Odra and probably in other rivers that open out into the North- and Baltic Sea; Danuberace (*A. a. a. pretzmanni*) in the upper part of the Danube and its tributary including the Drau; Southrace (*A. a. a. canadziae*) in the Save river catchment (tributary of the Danube in Croatia) and the lower part of the Danube (Romania, Serbia).

**Figure 1** Noble crayfish (*Astacus astacus*) collected in the river catchment of the Danube in Romania (Picture: T. Schikora).

### Threats to native crayfish

One-third to one-half of the crayfish species worldwide is threatened with population decline or extinction (Taylor 2002). Besides habitat deterioration, pollution, and other man-made environmental changes, invasive species and pathogens are major threats to the survival of European crayfish species. Therefore, the International Union for Conservation of Nature (IUCN) classifies noble crayfish as a vulnerable species with a decreasing population trend in the international Red List (IUCN 2012). Some national Red Lists even classify noble crayfish as an endangered species (e.g. Germany) or 'Critically Endangered' (e.g. Sweden). Noble crayfish is further included in the Bern Convention (Appendix III) and listed in the European Habitat Directive (Appendix V).

European crayfish species, in particular noble crayfish have been an object of trade, commerce and zoological studies throughout Europe for more than 2000 years (Skurdal and Taugbøl 2002). This has led to stocking of noble crayfish into numerous new localities. Translocations have been documented in central Europe

for centuries, whereas translocations are not known from southeastern Europe (see Albrecht *et al.,* 1983 for more details). This leads to an extensive mixing of crayfish on the one hand and to an overharvesting of local populations on the other hand.

Today, several pathogens (viruses, protozoans, bacteria, fungi) causing severe declines in crayfish populations are known (Edgerton *et al.,* 2004, Longshaw 2011). The most significant threat to European crayfish species is the Oomycete *Aphanomyces astaci* causing the crayfish plague. *A. astaci* was introduced to Europe via North American (hereafter 'American') crayfish species that were stocked into European waters to replace the decreasing number of native crayfish populations (Alderman 1996). *A. astaci* is an invasive, crayfish-specific parasite causing mass mortalities in susceptible European crayfish populations (e.g. Kozubíková *et al.,* 2008, Vrålstad *et al.,* 2011). After the crayfish plague eradicated most noble crayfish populations in Sweden in 2002, only 3% of the populations were left compared to 1900 (Bohman *et al.,* 2006). The pathogen is amplified in susceptible dying animals and spores are subsequently released into the water, usually leading to 100% mortality of the European crayfish present in the respective water body or large parts of it (OIE 2009). *A. astaci* is an obligate pathogen of freshwater crayfish, but can also be grown in laboratory cultures (Aldermann 1996). A co-evolution of a pathogen and the affected population may lead to a relatively stable host-parasite relationship through enhanced immune functioning by the natural host and virulence attenuation by the parasite (Edgerton and Jussila 2004). Therefore the pathogen can coexist with American crayfish species in such a balanced host-pathogen relationship. If American crayfish species are severely stressed or immunocompromised by other pathogens or environmental factors, they may succumb to the crayfish plague infection (Persson *et al.,* 1987; Söderhäll and Cerenius 1992).

**Figure 2** Narrow-clawed crayfish (*Astacus leptodactylus*) from the Romanian Danube. The dark melanisation indicates a crayfish plague infection. (Picture: L. Pârvulescu)

Probably, *A. astaci* was first introduced to Europe in the late 1850s. This is known as the first infection wave in which thousands of native crayfish populations were eradicated (Aldermann 1996). While the natural hosts of *A. astaci*, American freshwater crayfish species were not recorded during this first crayfish plague outbreak in Europe they were repeatedly introduced later (Aldermann 1996, Holdich *et al.,* 2009). Until recently it was believed that all American crayfish are carrier of *A. astaci* (e.g. "This crayfish species is a permanent carrier of the parasite and there are no *A. astaci*-free *P. leniusculus*"; Cerenius *et al.,* 2003). In ***Appendix 3***, I test the New NICS calico crayfish (*Orconectes immunis*) for an infection with *A. astaci*. A positive result would identify a fourth American crayfish as transmitter and support the concern that all American crayfish species in European waters are carriers of the crayfish plague pathogen. It was further believed that native crayfish populations suffer 100% mortality when infected with the pathogen. However, recent studies have shown that a coexistence of *A. astaci* and European crayfish species is in some cases possible (Jussila *et al.,* 2011; Viljamaa-Dirks *et al.,* 2011), supposedly due to a

lowered virulence of *A. astaci*. Furthermore, a coexistence could also be possible when the pathogen is absent in some populations. In ***Appendices 4 and 5*** I test native narrow-clawed crayfish (Figure 2) from the Romanian Danube for an infection with the crayfish plague agent. Narrow-clawed crayfish coexist with spiny-cheek crayfish (Figure 3) in the upper Romanian Danube (***Appendix 4***) and live in allopatry in the lower Danube (***Appendix 5***). In ***Appendix 6*** I investigate coexisting populations of noble crayfish and spiny-cheek crayfish. The prevalence of the crayfish plague has major implications on the conservation and management strategies of the threatened European crayfish species, and is critical for further reintroduction (Kozubíková *et al.,* 2009) or translocation attempts.

**Figure 3** The 'Old NICS' spiny-cheek crayfish (*Orconectes limosus*) and the 'New NICS' Calico crayfish (*Orconectes immunis*) coexist in the River Rhine (Picture: B. Dahelean).

## Genetic Methods

Due to the specific attributes of each molecular method, a combination of methods was necessary for this thesis to resolve the key questions. The phylogeographic analysis of noble crayfish was based on sequence variations of mitochondrial DNA (cytochrome oxidase subunit I (COI) and 16S rRNA) and length variations of microsatellites. Real-time polymerase-chain reaction (qPCR) was used for the verification of *A. astaci* in crayfish populations.

According to Avise (1987) mitochondrial DNA (mtDNA) is an ideal molecular system for phylogenetic analysis because it is distinctive, easy to isolate, has a simple genetic structure, evolves at a rapid rate and exhibits a straightforward mode of genetic transmission since mtDNA is maternally inherited. These features make mtDNA a widely used marker to reconstruct the phylogeographic history of species. It helps to uncover the ice age refuges and the colonization history of a species (Weiss 2002). When a molecular clock is known, a dated phylogenetic tree can be constructed. However, only little or no signal can be seen from sequences in bottlenecked populations that recently colonized an area (Hewitt 1999). Therefore, I additionally utilized nuclear microsatellite data to track the recent gene flow and to estimate the population diversity. The short stretches of repetitive microsatellite DNA evolve at a rapid rate when endogenous DNA polymerase in the cell makes a replication error and either mistakenly adds or removes a copy (DNA slippage). These stepwise mutations occur much more often than other types of mutation (e.g. single base substitutions in mtDNA).

In a sequence as well as in a microsatellite analysis, the first step after the DNA-extraction is the amplification of a specific DNA fragment using PCR. However, to detect small traces of DNA, a conventional PCR is not sufficient. Therefore, for the verification of *A. astaci* DNA in carrier crayfish tissues, I applied quantitative TaqMan® minor groove binder (MGB) qPCR. In contrast to a conventional PCR, beside the two species-specific primers, an additional species-specific probe has to anneal to the target DNA in order to get a positive signal. The TaqMan® MGB primer and probes bind on the minor groove of the DNA strain and are extremely sensitive to mismatches in the annealing sites of primers and

probe (Yao *et al.,* 2006). Today, this method is the most specific and sensitive method to test for the presence of *A. astaci* (Vrålstad *et al.,* 2009; Tuffs and Oidmann 2011).

# OBJECTIVES

The genetic structure of a species (e.g. areas of endemism, geographical patterns in species richness) is important for conservation prioritization. Since the genetic structure is the result of historic processes, I aimed to reconstruct the post-glacial recolonization of central Europe by the endangered noble crayfish (***Appendix 2***). Furthermore, I intended to identify refugial areas that are hotspots of genetic diversity (***Appendix 1 and 2***) and should receive special attention in species conservation. To address these questions I used two genetic data sets: i) sequence data of the mitochondrial cytochrome oxidase subunit I gene (***Appendix 1 and 2***), 16S rRNA (***Appendix 2***) and ii) species-specific microsatellites (***Appendix 2***). The results serve as a basis for conservation management of European freshwater crayfish.

Since the most significant threat to European crayfish species is the crayfish plague pathogen *A. astaci*, the distribution of the disease agent has to be considered before any management action can be taken. In this thesis I aimed to expand the knowledge of the distribution of *A. astaci* using qPCR. One goal was to reveal if the American calico crayfish, which increases its range in the River Rhine rapidly since a few years, is a carrier of *A. astaci* (***Appendix 3***). On the other side, the American spiny-cheek crayfish increased its range in the lower Danube. I intended to reveal its *A. astaci* carrier status and also the status of the native narrow-clawed crayfish in the lower Danube (***Appendix 4***), as well as the status of narrow-clawed crayfish in the Danube Delta (***Appendix 5***). Furthermore, I wanted to reveal mechanisms facilating the coexistence of European and American crayfish species in the same lake.

# THESIS OUTLINE

My thesis addresses the objectives with six manuscripts, compiled in the ***Appendices 1-6*** (Figure 4). Two manuscripts deal with the genetic structure of the European noble crayfish throughout Europe (***Appendices 1 and 2***) and four manuscripts address questions concerning the distribution of the crayfish plague in Germany, Poland and Romania (***Appendix 3 to 6***).

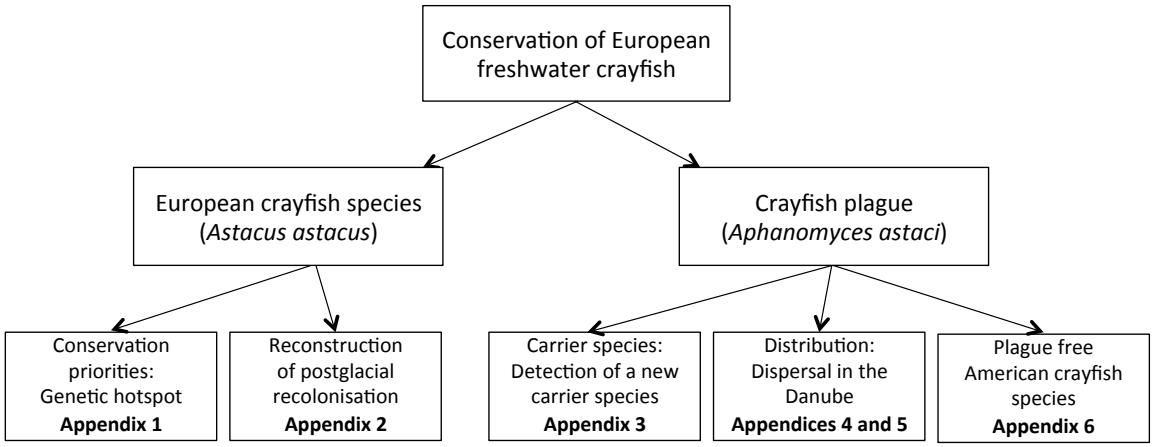


**Figure 4** Overview of the different Appendices of this thesis assigned to the two topics of my PhD work: European crayfish species and crayfish plague.

## PART 1 - European crayfish species (*Astacus astacus*)

The conservation of genetic diversity is one goal in modern conservation strategies because genetic variation represents an advantage for the long-term survival of species. To protect the within-species diversity, a good knowledge of the genetic structure of this species is indispensable. Since the genetic structure of a species is a result of both present processes and past history, the post-glacial recolonization history should be reconstructed in order to reveal the genetic structure of a species.

Appendix 1: **First large-scale genetic analysis of the vulnerable noble crayfish *Astacus astacus* reveals low haplotype diversity of central European populations**

In Appendix 1 I present the first study focusing on the genetic structure of noble crayfish throughout its European distribution area. I analyzed a partial sequence of the mitochondrial gene cytochrome oxidase subunit I (COI) from a large set of noble crayfish populations throughout Europe to localize genetic hotspots and to prioritize conservation effort. This study has shown that the highest haplotype diversity is located in southeastern Europe, which indicates with reasonable certainty a glacial refugium. In contrast, I showed that diversity is relatively low in central Europe, which was recolonized after the last ice age. I discovered differentiation between river catchments despite the extensive human translocation of crayfish. In this first study I revealed that more markers are needed to better understand the recolonization history of noble crayfish. Furthermore, I recognized areas from where more samples are needed in order to identify the genetic hotspots more specifically in a subsequent supplementary study.

Appendix 2: **Recolonization of Europe by noble crayfish (*Astacus astacus*) – natural versus human-mediated processes**

In the second Appendix I focus on the postglacial recolonization of central Europe by noble crayfish. For this, I improved the molecular methods by additionally using nuclear markers (microsatellites) compared to the previously performed mtDNA-based study. Additionally, 16S rRNA was used as a second mitochondrial marker besides COI. By using these methods, I was able to calculate the first calibrated tree of noble crayfish and correlate the split of lineages with climatological events. I detected a very distinct lineage in the Western Balkans that may have served as an isolated glacial refugium during the last glacial maximum. I suppose a second independent refugium in the eastern Black Sea basin from where the species recolonized central Europe through the Danube and through a second migratory route in eastern Europe.

**PART 2 - Crayfish plague (*Aphanomyces astaci*)**

For the conservation of European crayfish species, knowledge on the distribution of *A. astaci* in European waters is of great importance. This aspect correlates with the question which crayfish species are latent carrier of the disease agent.

Appendix 3: **Crayfish plague agent detected in populations of the invasive North American crayfish *Orconectes immunis* (Hagen, 1870) in the Rhine River, Germany**

In the third Appendix I identified calico crayfish as the fourth North American crayfish species in European waters, which is carrier of the crayfish plague pathogen. A positive verification was received from two sampling sites in the upper Rhine River where calico crayfish seems to displace its invasive predecessor spiny-cheek crayfish. This finding of the positive detection is worrying considering the fast and successful invasion process of calico crayfish. Furthermore, this outcome supports the apprehension that all American crayfish species are carriers of the crayfish plague.

Appendix 4: **Invasive crayfish and crayfish plague on the move: first detection of the plague agent *Aphanomyces astaci* in the Romanian Danube**

The fourth Appendix focuses on the dispersal of *A. astaci* in the Romanian Danube. The crayfish plague disease agent could be detected in American narrow-clawed crayfish as well as in European spiny-cheek crayfish. According to field observations of the first occurrences of narrow-clawed crayfish along the Danube, an invasion speed could be calculated for this species. It was estimated that the invasive narrow-clawed crayfish invade the sensitive Danube Delta area in the mid-2060s, but the crayfish plague agent may reach the delta significantly earlier.

Appendix 5: **Crayfish plague pathogen detected in the Danube Delta – a potential threat to freshwater biodiversity in southeastern Europe**

In the fifth Appendix I survey native narrow-clawed crayfish from the highly protected Danube Delta for an infection with *A. astaci*, with a positive test result. The delta is located 970 km downstream of the current invasion front of American

crayfish in the Danube. This result shows that the pathogen has a much wider range in this river than previously assumed. *A. astaci* seems to persist in local populations, as neither crayfish mass mortalities, nor alien crayfish species have been reported from this region. In this Appendix different possible dispersal ways of the pathogen towards the delta are discussed. However, the real transmitter/vector could not be determined.

Appendix 6: **Absence of the crayfish plague pathogen (*Aphanomyces astaci*) facilitates coexistence of European and American crayfish in central Europe**

In the sixth Appendix I study coexisting populations of introduced spiny-cheek crayfish and native noble crayfish. I resolve the question whether the coexistence resulted from reduced virulence in local *A. astaci* strains, increased resistance in susceptible crayfish species, or a complete absence of the pathogen in the American crayfish populations. A negative result of 523 crayfish tested for an infection with *A. astaci* is a very strong indication that the coexistence is possible in the absence of the pathogen in these populations. Exposure experiments confirmed these results.

# SUMMARY OF RESULTS

In this chapter I will briefly summarize the most important results of my thesis. In the following discussion I will get back to these results. For more detiails on the results see the Appendices.

## *European crayfish species (Astacus astacus)*

In the first part using sequence analyses and microsatellite analyses I revealed high genetic diversities (high number of haplotypes and number of alleles) of noble crayfish in southeastern Europe (***Appendix 1 and 2***). The haplotype diversity ($H_D$) is highest in the Black Sea basin ($H_D$ = 0.851, Table 2). In contrast the genetic diversities are low in central Europe. The lowest haplotype diversity was detected in the Baltic Sea basin ($H_D$ = 0.276). I detected the highest number of differentiated haplotypes/alleles in the Western Balkans (Adriatic Sea basin and south-western Black Sea basin; red Star in Figure 6). Six haplotypes from the Western Balkans (Hap41 – Hap46) were highly distinct from all other haplotypes (Figure 2 in ***Appendix 2***). The calibrated Bayesian phylogenetic tree revealed that the Western Balkan haplotypes (Lineage 4 in Figure 5) were separated from all other haplotypes about 710,890 years before present (YBP), whereas all other differentiations occurred within the last 450,000 YBP. The microsatellite analysis supported the differentiation of the Western Balkan samples (Figure 5 in ***Appendix 2***).

**Table 2** Results from a combined 350 base pair (bp) fragment of the COI sequence and a 476 bp fragment of the 16S rRNA sequences for the analyzed sea basins. The number of sequenced individuals (N), the number of haplotypes ($H_N$) and the haplotype diversity ($H_D$) is given (also see ***Appendix 2***).

| | Sites | N | $H_N$ | $H_D$ |
|---|---|---|---|---|
| Baltic Sea | 14 | 66 | 5 | 0.276 |
| North Sea | 59 | 249 | 13 | 0.316 |
| Black Sea | 82 | 185 | 28 | 0.851 |
| Adriatic Sea | 5 | 12 | 5 | 0.576 |

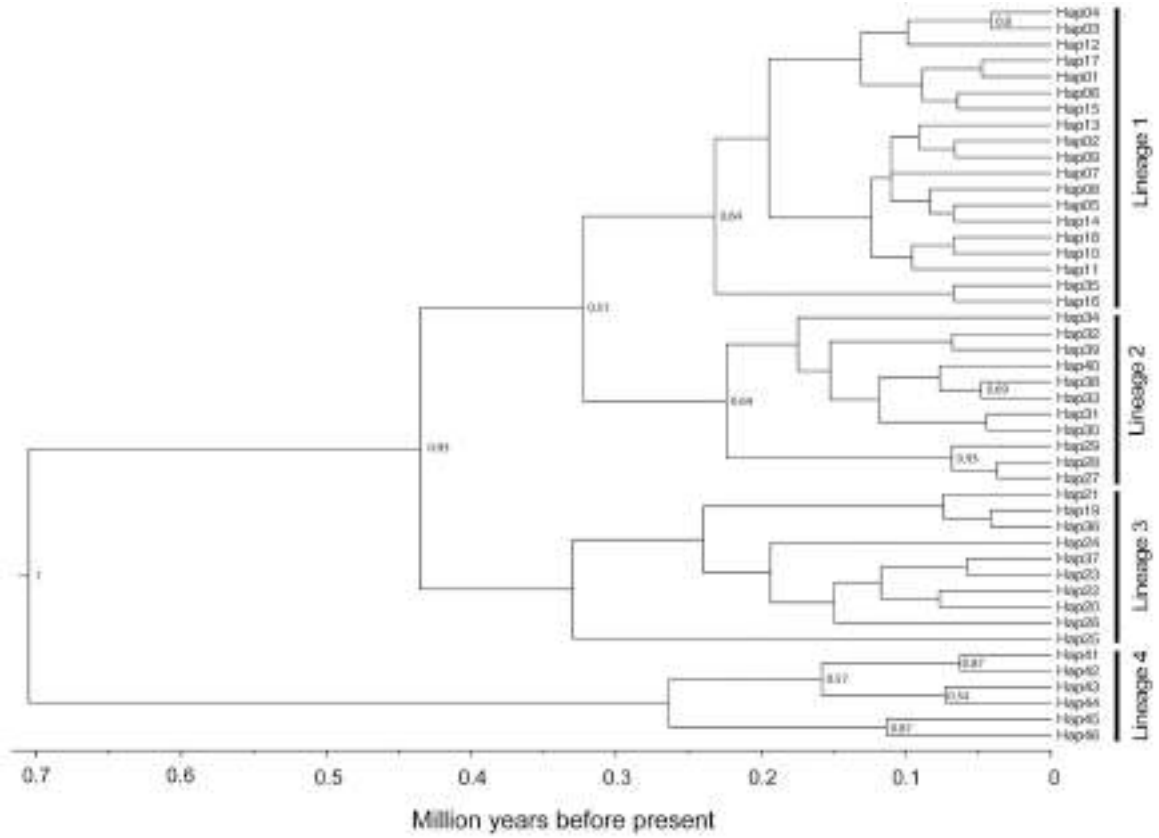


**Figure 5** Phylogenetic tree generated using Bayesian Markov Chain Monte Carlo analysis implemented in Bayesian Evolutionary Analysis Sampling Trees (BEAST; Drummond *et al*., 2012) by using a combined fragment of the COI sequence and a fragment of the 16S rRNA sequence. The axis shows the time scale in million years before present. Shown are the posterior probability values above 0.5 (see also ***Appendix 2***).

In total, eight haplotypes were shared between different sea basins and between different river catchments. For example the haplotype Hap41 from the differentiated lineage 4 was shared between the Danube river catchment in the Western Balkans and the Elbe river catchment (North Sea basin).

The probability values/Bayes factors for the model selection calculated with Migrate-n version 3.3.2 (Beerli and Felsenstein 2001) using mtDNA sequence data preferred model 2b (see ***Appendix 2***). This model implies a 'bifurcal' colonization route for noble crayfish from the eastern Black Sea basin (Romania, Bulgaria) to a) the North Sea basin and to b) the Baltic Sea basin (see schematic arrows in Figure 6).

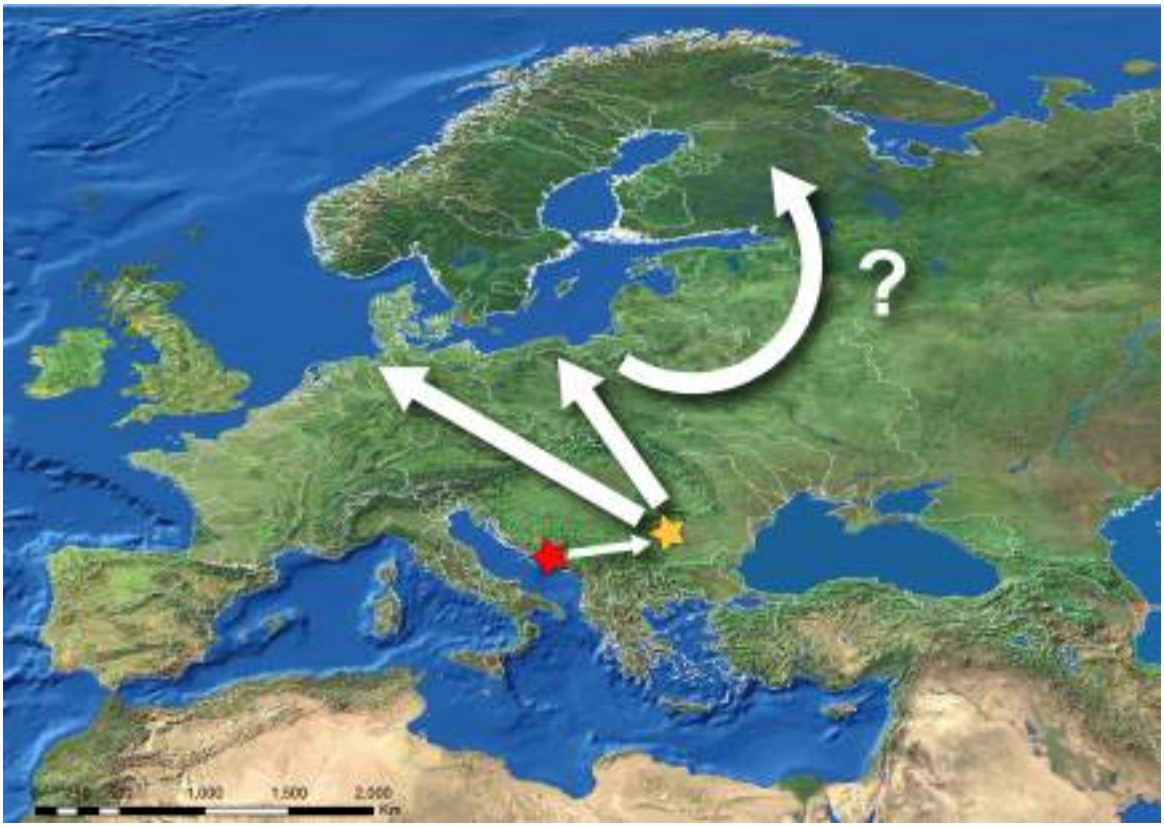


**Figure 6** Hypothetical migration routes of noble crayfish from its glacial refugia in southeastern Europe into northern and central Europe are shown schematically. Arrows indicate the directions of gene-flow. Hotspots of genetic diversity are indicated with stars. Red star: Western Balkan, yellow star: eastern Black Sea basin. The '?' indicates that the route to Finland should be considered with caution because of the low sample size of Finnish samples (N = 10) (see also ***Appendix 2***).

### Crayfish plague (*Aphanomyces astaci*)

In ***Appendix 3*** using qPCR I tested the New NICS calico crayfish from the river Rhine in Germany positive for an infection with *A. astaci*. From the sampling site Germersheim in the main river 60% of the samples were positive (N = 50; $N_+$ = 23) and from Bühl (a side channel of the Rhine) 81% were positive (N = 32; $N_+$ = 26). With this positive verification I identified calico crayfish as the fourth American crayfish species to be carrier of the crayfish plague agent (***Appendix 3***).

Furthermore I detected the crayfish plague pathogen in American spiny-cheek crayfish and in native narrow-clawed crayfish in the lower Danube in Romania (***Appendix 4***). The pathogen could be detected in 41% of the tested native species (N = 49; $N_+$ = 20) and in 32% of the tested NICS (N = 71; $N_+$ = 23). Moreover, I detected that native narrow-clawed crayfish in the Danube Delta at

two sampling sites about 970 km downstream of the current invasion front of American crayfish in the Danube is a carrier of *A. astaci* (***Appendix 5***). In total, 30% of the samples from the Chilia Channel (N = 37; $N_+$ = 11) and 67% of the samples from the Merhei Lake (N = 3; $N_+$ = 2) were tested positive.

On the other side, from a total of 523 tested crayfish (490 spiny-cheek crayfish, 33 noble crayfish) from six coexisting populations of European and American crayfish species, no *A. astaci* positive individuals were found. With an assumed lowest possible *A. astaci* prevalence of 10% in a carrier population, there is a 98% probability of disease absence in five of the nine tested coexisting populations. Exposure experiments confirm these results: no abnormal mortality or behavioural changes were seen in noble crayfish kept together with American crayfish from the coexisting populations.

# GENERAL DISCUSSION

In the following discussion I will first address the two parts of my thesis (native crayfish and crayfish plague) separately and after that I will discuss these aspects that are inextricably linked together combined.

## Conservation considerations - native crayfish

The results of this thesis indicate that special attention in species conservation of noble crayfish should be put on the Danube river catchment in southeastern Europe, where the highest genetic diversity was detected (***Appendix 1***, Schrimpf *et al*., 2011). Noble crayfish outlasted cold climate phases in southeastern Europe while the climate conditions in central and northern Europe were unfavorable for crayfish species. The same applied to many other freshwater species (e.g. Hänfling *et al.,* 2009; Weiss *et al.,* 2002). In particular, I detected that noble crayfish outlasted the last glacial maximum in at least two different refugia in southeastern Europe: one was located in the Western Balkans and one in the eastern Black Sea basin (see scheme in Figure 6). Noble crayfish from the Western Balkans are particularly diverse and highly differentiated. While the Western Balkan haplotypes (Lineage 4 in Figure 5) were separated from all other haplotypes already about 711,000 YBP, all other haplotypes differentiated within the last 450,000 YBP. Therefore, I assume that the Western Balkans served as an older glacial refuge (***Appendix 2***, Schrimpf *et al*., unpublished data). Further, the high diversity in the eastern Black Sea basin and the similarity to central European haplotypes indicates a second refuge in the Danube river catchment from where the noble crayfish recolonized central Europe. It has to be mentioned, that the data from the eastern Black Ses basin is restriced to Romania and Bulgaria. However, it is very well possible that the glacial refuge was even further south, e.g. in Greece, and Romania and Bulgaria were recolonized from a more southern refuge before noble crayfish recolonized central and northern Europe from the eastern Black Sea basin. I revealed that the recolonization of the North Sea basin was possible along the Danube and the recolonization of the Baltic Sea basin along a second eastern corridor. The sample size of Finnish

samples however was too small (N = 10) to definitely reveal the colonization route of Finland. In an additional study more samples from eastern and northern Europe should be analyzed in order to reveal the eastern corridor more specific and to reconstruct the (re-)colonization of northern Europe.

In this thesis I have detected that the genetic structure of noble crayfish is partly dissolved (***Appendices 1 and 2***). This altered genetic structure was probably caused by the high anthropogenic influence on the noble crayfish dispersal (e.g. translocation) throughout Europe (Skurdal and Taugbøl, 2002). The haplotype Hap41 from the differentiated Lineage 4 that was detected in the Western Balkans and in the Elbe river catchment (***Appendix 2***), was probably introduced to the Elbe river catchment artificially and led to a 'genetic contamination' of the local population (Largiadèr *et al*., 2000). Long-distance translocation of noble crayfish needs to be prohibited to conserve the remaining (natural) genetic structure of the species. However, the disrupted genetic structure might explain why partly different and contradictory classifications of the noble crayfish subspecies based on morphological criteria can be found in the literature (see Smietana *et al.,* 2006 for a review). When noble crayfish are collected for morphological determination from a specific sampling site, it is very well possible that this site was stocked artificially and that researchers did therefore not always use autochthonous crayfish to define morphological characteristics of noble crayfish for a certain region.

The geographical assignment of the three races of *A. a. astacus* based on morphological characters (Karaman 1962, 1963, see also the Introduction of my thesis) cannot be confirmed with the genetic data of individuals from the same regions (***Appendices 1 and 2***). It was not possible to reveal if the discrepancy between morphological and genetic differentiation can be explained by a difference between morphological and genetic characters (e.g. morphological and genetic characters evolve at a different rate) or by artificial translocations. Presumably, it is a combination of different aspects.

However, as long as the taxonomic status of a species is not resolved, one cannot be sure which taxonomic unit is being translocated: a different species, a subspecies, a race or a genetic variation. It is oftentimes impossible to define the

taxonomic level, since the threshold for any taxonomic level (e.g. species, subspecies, race) is difficult to define. The existence of different species concepts additionally complicates the delimitation of a species/subspecies (de Queiroz 2007). A species under the 'Biological species concept' (sensu Mayr 1942, a species is defined based on its ability to inbreed) could be considered a subspecies under the 'Phylogenetic species concept' (Cracraft 1983) or vice versa. Therefore, any introduction of individuals from a distant region needs to be prohibited and the conservation of local population should be promoted in order to protect local adaptions and endemic haplotypes/alleles. This way the greatest possible genetic variation of a species can be conserved.

The taxonomic status of European crayfish species is not fully resolved (Holdich *et al*., 2006). One example is the white-clawed crayfish case (*A. pallipes* species complex), whose taxonomic order is still under revision. While in the past A. pallipes was considered a 'species', it is today considered a 'species complex' (Holdich *et al.,* 2006). In the future the *A. pallipes* 'species complex' might be split into two separate species (*A. pallipes* and *A. italicus*), as proposed by Grandjean *et al.,* (2002). With regard to noble crayfish, we cannot exclude, that populations today known as noble crayfish throughout Europe will in future be classified as more than one species. Only if between-catchment translocation of noble crayfish is stopped and translocation for conservation purposes will be conducted within ESUs (e.g. river catchments or even smaller units, like side arms of a river), the conservation of the species integrity is possible. In summary, there is urgent need for a close collaboration between taxonomists and molecular biologists as well as conservation biologists to reveal the taxonomic status of European crayfish species as a basis for species conservation.

Unfortunately, the region with the highest genetic diversity of noble crayfish coincides with the region that is under current threat of disease carrying American crayfish species (***Appendices 4 and 5***, Hudina *et al.,* 2009, Pârvulescu *et al.,* 2009). Therefore, before any conservation measures of native crayfish can be conducted the distribution of the crayfish plague pathogen has to be considered.

## *Conservation considerations - crayfish plague*

Until recently it was believed that a) all (North) American crayfish are carrier of *A. astaci* (e.g. "This crayfish species is a permanent carrier of the parasite and there are no *A. astaci*-free *P. leniusculus*"; Cerenius *et al.,* 2003) and that b) native crayfish populations suffer 100% mortality when infected with *A. astaci* (Table 3). With the positive verification of calico crayfish as carrier of *A. astaci* (***Appendix 3***, Schrimpf *et al*., 2013) I support the assumption a) that all American crayfish *spesies* are indeed potential carriers. However, I discovered potential plague-free spiny-cheek crayfish populations and therefore I show that not all *populations* of American crayfish species in Europe are necessarily infected with *A. astaci* (***Appendix 6***, Schrimpf *et al*., in press). These results demonstrate that the disease transmission risk varies substantially between different American crayfish populations. The existence of plague-free American crayfish populations has been assumed by several authors (e.g. Westman and Savolainen, 2001, Jussila and Mannonen 2004, Skov *et al.,* 2011) but never before been studied thoroughly. In addition, I contradict the assumption b) that all European crayfish suffer 100% mortality when infected with *A. astaci*. With the positive verification of narrow-clawed crayfish as carrier of the agent of the crayfish plague (***Appendices 4 and 5***, Pârvulescu *et al*., 2012, Schrimpf *et al*., 2012), I show that latent infected native crayfish populations do exist. This result is supported by recent molecular studies, which also indicate that persistent infections of *A. astaci* occur in native European crayfish (Jussila *et al.,* 2011; Viljamaa-Dirks *et al.,* 2011; Kokko *et al.,* 2012, Svoboda *et al*., 2012).

**Table 3** The common assumption and the outcome of this thesis regarding the infection status of North American crayfish populations (NACP) and the reaction of European crayfish population (ECP) to an infection. ✔ = correct, ✗ = wrong.

| | **Common assumption** | | **This thesis** | |
|---|---|---|---|---|
| | **All populations are infected** | **All populations die shortly after infection** | **All populations are infected** | **All populations die shortly after infection** |
| NACP | ✔ | ✗ | ✗ | ✗ |
| ECS | ✗ | ✔ | ✗ | ✗ |

These findings demonstrate that even after many years of visual observations, one cannot know if *A. astaci* is present or absent in a crayfish population. The wrong assumption of the plague´s absence can have fatal consequences for other native crayfish populations because the pathogen must not be equally lethal to all populations. It is possible that some local populations evolved a resistance against this pathogen, but other recently infected populations without previous 'selection opportunity' could be susceptible and might be completely destroyed. Therefore, before native crayfish individuals are being translocated to a new locality, molecular analyses need to be done to reveal the carrier status of the donor population. On the other side, European crayfish populations are not necessarily lost, in case American crayfish occur in the same water. The native crayfish can be transferred to waters that are free of American crayfish species. Furthermore, it has to be considered that any translocation also involves the risk of spreading other diseases and associated non-target species 'accidentally' (Edgerton and Jussila 2004).

American crayfish species were initially introduced into Europe to replace native crayfish that suffered high mortalities due to environmental changes (Alderman *et al.,* 1990). Back then, the replacement of one species by another one, which occupies a similar ecological niche, was not believed to cause any negative ecological consequences. However, today we know that there are differences in the dietary habits between different crayfish species and that their physical impact on the environment varies (see Holdich 2002b for a summary of different species). Thus, the replacement of one crayfish species by another can have fatal consequences on the environment. For this reason, nowadays the introduction of American crayfish species into the wild in Europe is forbidden and the western European native species (*A. astacus*, *A. pallipes*, *A. torrentium*) are listed as protected in Appendix III of the Bern Convention. Species listed under Appendix III are those who are in need of protection and any exploition of wild fauna shall be regulated in order to keep the populations out of danger (Council of Europe 2009).

## *Synthesis of discussion*

Protection of the native biodiversity is the major aim of conservation biology (Frankham *et al*. 2004). In the last decades many conservation projects have been conducted that consisted in restoring the habitat, (re-)introducing native crayfish species (Taugbøl and Peay 2004, Dümpelmann 2011) or eradicating NICS in the wild (Sandodden and Johnsen 2010). However, while there are numerous reports on successful conservation measures, many introductions have failed due to new crayfish plague outbreaks. The extinction of local populations can have fatal consequences for the environment. Due to their trophic activities as omnivores crayfish play a key role in many freshwater ecosystems (Nyström 1999), and their loss may change the food-web with drastic impacts on local species composition. As long as infected crayfish species are subject to aquarium trade, NICS and the crayfish plague pathogen will repeatedly be introduced into the wild. Many people promote the introduction of exotic species because trading these species promises increased profit (McNeely 2001). The persons involved in the introduction often don't consider the negative impact these species might have on the environment. Usually it's the general public and future generations who have to pay the price.

Germany is the main importer of exotic crayfish species in Europe (Chucholl 2012). The majority of the 120 NICS, which are available in German aquarium trade originate from America and is therefore suspected to be carrier of the crayfish plague. Contrary, in Norway the import of live crayfish is banned and until recently Norway was free of NICS (Vrålstad *et al.,* 2011). However, few crayfish plague outbreaks and the recent findings of NICS in Norway (Johnsen *et al*., 2007, Vrålstad *et al*., 2011) are believed to have resulted from introductions of NICS and transmissions of *A. astaci* from lakes in Sweden close to the Norwegian border (Taugbøl 2004). Boats or fishing gear used on both sides of the border might have served as vectors for the *A. astaci* transmission. This example highlights that all European countries have a responsibility not only for their own environment but also for that of their neighbouring countries because non-indigenous species do not adhere to national boundaries (European Commission 2008).

# CONCLUSION

The results of my studies show that a high degree of genetic diversity of noble crayfish can be conserved when the genetic hotspots in southeastern Europe receive special attention in conservation management (***Appendices 1 and 2***). Genetic diversity and endemic haplotypes/alleles can be protected when species introduction for conservation purposes is being conducted only within ESUs (river catchments) and translocations between river catchments are strictly avoided. However, the greatest threat to native crayfish biodiversity stems from the introduction of NICS infected with crayfish plague (Lodge *et al.,* 2000). Because the disease agent may devastate conservation measures, its distribution has to be considered before any management action can be taken. In my thesis I could verify *A. astaci* in several populations in Poland, Germany and Romaia in native and American crayfish species (***Appendices 3 - 6***). While I could show that plague-free American crayfish populations do exist (***Appendix 6***) and native Euroipean crayfish can be infected without indication of illness (***Appendices 3 and 4***), these findings however constitute exceptions. Moreover, the results show that even native species may serve as a transmitter between water bodies even if they don't show signs of infection. Therefore, a health test should be conducted before native crayfish species are translocated.

As long as infected NICS can be purchased by anybody via pet stores or internet, they will repeatedly be introduced into the wild and eradicate genetic resources of native crayfish. NICS and the crayfish plague continue to be the greatest threat to the survival of native crayfish. Therefore, crayfish conservation needs to address the threat posed by NICS with a high priority. A change in legislation, which prohibits the trade with infected invasive species will increase the long-term survival of native crayfish species and is therefore urgently needed.

# REFERENCES


Agerberg A (1990) Genetic variation in three species of freshwater crayfish; *Astacus astacus* L., *Astacus leptodactylus* Aesch., and *Pacifastacus leniusculus* (Dana), revealed by isozyme electrophoresis. Hereditas, 113: 101–108.

Alaranta A, Henttonen P, Jussila J, Kokko H, Prestegaard T, Edsman L and Halmekyto M (2006) Genetic differences among noble crayfish (*Astacus astacus*) stocks in Finland, Sweden and Estonia based on the ITS1 region. Bull Fr Pêche Piscic, 380-381: 965–975.

Albrecht H (1983) Besiedlungsgeschichte und ursprüngliche holozäne Verbreitung der europäischen Flußkrebse (Decapoda: Astacidae). Spixiana, 6: 61-77.

Alderman DJ, Holdich DM and Reeve I (1990) Signal crayfish as vectors in crayfish plague in Britain. Aquaculture, 86: 3–6.

Alderman DJ (1996) Geographical spread of bacterial and fungal diseases of crustaceans. Revue Scientifique Et Technique - Office International Des Epizooties, 15: 603–632.

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, *et al.* (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu Rev Ecol Syst, 18: 489-522.

Axelsson E, Nyström P, Sidenmark J and Brönmark C (1997) Crayfish predation on amphibian eggs and larvae. Amphibia-Reptilia 18: 217–228.

Beerli P and Felsenstein J (2001) Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. Proc Natl Acad Sci USA, 98: 4563–4568.

Bertocchi S, Brusconi S, Gherardi F, Grandjean F and Souty-Grosset C (2008) Genetic variability of the threatened crayfish Austropotamobius italicus in Tuscany (Italy): implications for its management. Fund Appl Limnol, 173: 153–164.

Bij de Vaate A, Jazdzewski K, Ketelaars HAM, Gollasch S, Van der Velde G (2002) Geographical patterns in range extension of Ponto-Caspian macroinvertebrate species in Europe. Canadian Journal of Fisheries and Aquatic Sciences 59: 1159-1174

Bohman P, Nordwall F and Edsman L (2006) The effect of the large-scale introduction of signal crayfish on the spread of crayfish plague in Sweden. Bull Fr Pêche Piscic, 380-381: 1291-1302.

Cracraft, J (1983) Species Concepts and Speciation Analysis. Cur Ornith, 1: 159-187.

Cerenius L, Bangyeekhun E, Keyser P, Söderhäll I, Söderhäll K (2003) Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci.* Cell Microbiol 5: 353−357.

Chucholl C (2012) Invaders for sale: trade and determinants of introduction of ornamental freshwater crayfish. Biol Invasions 15: 125–141.

Chucholl C and Pfeiffer M (2010) First evidence for an established Marmorkrebs (Decapoda, Astacida, Cambaridae) population in Southwestern Germany, in syntopic occurrence with *Orconectes limosus* (Rafinesque, 1817). Aquat Invasions 5: 405–412.

Council of Europe (2009) Convention on the Conservation of European Wildlife and Natural Habitats, www.conventions.coe.int, (accessed 30 December 2012).

Correia AM and Ferreira O (1995) Burrowing Behavior of the Introduced Red Swamp Crayfish *Procambarus Clarkii* (Decapoda, Cambaridae) in Portugal. J of Crustacean Biol 15: 248–257.

Crandall KA (2006) Applications of phylogenetics to issues in freshwater crayfish biology. Bull Fr Pêche Piscic, 380-381: 953-964.

Crandall KA and Buhay JE (2011) Global diversity of crayfish (Astacidae, Cambaridae, and Parastacidae – Decapoda) in freshwater. Hydrobiologia, 595: 295–301.

de Queiroz, K (2007) Species concepts and species delimitation. Syst Biol, 56(6): 879–886.

Donoghue MJ (1985) A Critique of the Biological Species Concept and Recommendations for a Phylogenetic Alternative. The Bryologist, 88(3): 172-181.

Drummond AJ, Suchard MA, Xie D and Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol, 29: 1969-1973.

Dudgeon D, Arthington AH, Gessner MO, Kawabata ZI, Knowler DJ, *et al*. (2006) Freshwater biodiversity: importance, threats, status and conservation challenges. Biol Reviews, 81: 163–182.

Dümpelmann C (2011) Konzeption und Vorgehensweise bei der Erfassung von Krebsbeständen in größeren Einzugsgebieten – Erfahrungen aus dem hessischen Lahnsystem. 5. Internationales Flusskrebsforum 2011. Tagungsband. pp 21-26.

Durand JD, Persat H and Bouvet Y (1999) Phylogeography and postglacial dispersion of the chub (*Leuciscus cephalus*) in Europe. Mol Ecol, 8: 989–997.

Edgerton BF and Jussila J (2004). Keynote presentation and roundtable session 4, Crayfish pathology in Europe: past, present and a programme for the future. Bull Fr Pêche Piscic, 372-373(1-2): 473-48.

Edgerton BF, Henttonen P, Jussila J, Mannonen *A*, Paasonen P, Taugbøl T, *et al*. (2004) Understanding the causes of disease in European freshwater crayfish*.* Conserv Biol*,* 18: 1466–1474.

Edsman L, Farris JS, Kallersjo M and Prestegaard T (2002) Genetic differentiation between noble crayfish, *Astacus astacus* (L.) populations detected by microsatellite length variation in the rDNA ITS1 region. Bull Fr Pêche Piscic, 367: 691–706.

Edsman L, Füreder L, Gherardi F and Souty-Grosset C (2010) *Astacus astacus*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. Available at: www.iucnredlist.org (accessed 30 October 2012).

European Commission (2008) Developing an EU Framework for Invasive Alien Species: Discussion Paper (Final), available from http://ec.europa.eu/environment/nature/invasivealien/docs/ias_discussion_paper.pdf (accessed 10 December 2012).

Fevolden SE, Taugbøl T and Skurdal J (1994) Allozymic variation among populations of noble crayfish, *Astacus astacus* L., in southern Norway: implications for management. Aquacult Fish Manage, 25: 927–935.

Frankham R, Ballou JD and Briscoe DA (2004). A primer of conservation genetics. Cambridge University Press, Cambridge, 234 pp.

Füreder L, Gherardi F, Holdich D, Reynolds J, Sibley P and Souty-Grosset C (2010) *Austropotamobius pallipes*. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. Available at: www.iucnredlist.org. (accessed 30 October 2012).

Gouin N, Grandjean F and Souty-Grosset C (2006) Population genetic structure of the endangered crayfish *Austropotamobius pallipes* in France based on microsatellite variation: biogeographical inferences and conservation implications. Freshwater Biol, 51: 1369–1387.

Grandjean F, Frelon-Raimond M and Souty-Grosset C (2002) Compilation of molecular data for the phylogeny of the genus Austropotamobius*:* one species or several? Bull Fr Pêche Piscic, 367: 671-680.

Guan R, and PR Wiles (2002) Ecological impact of introduced crayfish on benthic fishes in a British lowland river. Conservation Biology 11(3): 641–647.

Hänfling B, Dümpelmann C, Bogutskaya NG, Brandl R and Brandle M (2009) Shallow phylogeographic structuring of *Vimba vimba* across Europe suggests two distinct refugia during the last glaciation. J Fish Biology 75*,* 2269–2286.

Hewitt GM (1999) Post-glacial re-colonization of European biota. Biol J Linn Soc, 68: 87–112.

Holdich DM (2002a). Background and functional morphology. In: Holdich DM (ed), Biology of freshwater crayfish. Blackwell Science, Oxford, United Kingdom, pp 3–29.

Holdich DM (ed.) (2002b) Biology of Freshwater Crayfish, Blackwell Science, Oxford, United Kingdom, 512 pp.

Holdich DM, Haffner P, Noël P (2006) Species files. In: Souty-Grosset C, Holdich DM. Noël PY, Reynolds JD, Haffner P (eds), Atlas of Crayfish in Europe. Muséum national d'Histoire naturelle, Paris, (Patrimoines naturels, 64), pp 76-81.

Holdich DM, Reynolds JD, Souty-Grosset C, Sibley PJ (2009) A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. Knowl Managt Aquatic Ecosyst, 11: 394–395.

Hudina S, Faller M, Lucić A, Klobučar G, Maguire I (2009) Distribution and dispersal of two invasive crayfish species in the Drava River basin, Croatia. Knowl Manag Aquat Ecosyst 394−395: 9.

IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. Available at: www.iucnredlist.org (accessed 10 December 2012).

Johnsen SI, Taugbol T, Andersen O, Museth J, Vrålstad T(2007) The first record of the non-indigenous signal crayfish *Pasifastacus leniusculus* in Norway. Biol Invasions 9: 939−941.

Jussila J and Mannonen A (2004) Crayfisheries in Finland, a short overview. Bull Fr Pêche Piscic, 372–373(2): 263–274.

Jussila J, Makkonen J, Vainikka A, Kortet R and Kokko H (2011) Latent crayfish plague (*Aphanomyces astaci*) infection in a robust wild noble crayfish (*Astacus astacus*) population. Aquaculture 321: 17–20.

Karaman MS (1962) Ein Beitrag zur Systematik der Astacidae (Decapoda). Crustaceana, 3: 173–191.

Karaman MS (1963) Studie der Astacidae (Crustacea, Decapoda) II. Teil. Hydrobiologia, 22: 111–132.

Kokko H, Koistinen L, Harlioğlu MM, Makkonen J, Aydın H and Jussila J (2012) Recovering Turkish narrow-clawed crayfish (*Astacus leptodactylus*) populations carry *Aphanomyces astaci*. Knowl Managt Aquatic Ecosyst, 404: 1-7.

Kozubíková E, Filipová L, Kozák P Ďuriš Z and others (2009) Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in invasive American crayfishes in the Czech Republic. Conserv Biol, 23: 1204−1213.

Kozubíková E, Petrusek A, Ďuriš Z, Martín MP, Diéguez-Uribeondo J and Oidtmann B (2008) The old menace is back: recent crayfish plague outbreaks in the Czech Republic. Aquaculture, 274: 208−217.

Lodge DM, Taylor CA, Holdich DM and Skurdal J (2000) Nonindigenous crayfishes threaten North American freshwater biodiversity*:* Lessons from Europe. Fisheries, 25(8): 7–20.

Largiadèr CR, Herger F, Lörtscher M and Scholl A (2000) Assessment of natural and artificial propagation of the white-clawed crayfish (*Austropotamobius pallipes* species complex) in the Alpine region with nuclear and mitochondrial markers. Mol Ecol, 9: 25–37.

Longshaw M (2011) Diseases of crayfish: a review. J Invertebr Pathol, 106: 54−70.

Nyström P (1999) The effects of crayfish on interactions in freshwater benthic communities. PhD thesis, Lund University, Lund, Sweden.

Malcolm L, Hunter Jr, and James P (2007) Fundamentals of Conservation Biology. 3rd edition. Blackwell Publishing, Oxford, United Kingdom, pp 516.

Mayr E (1942) Systematics and the Origin of Species from the Viewpoint of a Zoologist. Cambridge, Mass., Harvard University Press, 1999. pp 334. Originally published: New York : Columbia University Press, 1942.

McGeoch MA, Butchart SHM, Spear D, Marais E, Kleynhans EJ, Symes A, Chanson J and Hoffmann M (2010) Global indicators of biological invasion: species numbers, biodiversity impact and policy responses. Divers Distrib, 16: 95–108.

McNeely J (2001) Invasive species: a costly catastrophe for native biodiversity. Land Use and Water Resources Research 1: 1–10.

Moritz C (1994) Defining 'evolutionary significant units' for conservation. Trends Ecol Evol*,* 9: 373–375.

OIE (Office international des épizooties) (2009) Crayfish plague (*Aphanomyces astaci*). Chapter 2.2.1. In: Manual of diagnostic tests for aquatic animals, 6th edition. Office international des épizooties, Paris, p 63-77. Available at: www.oie.int (accessed 12 December 2012).

Pârvulescu L, Paloș C, Molnar P (2009) First record of the spiny-cheek crayfish *Orconectes limosus* (Rafinesque, 1817) (Crustacea: Decapoda: Cambaridae) in Romania. North-West J Zool 5:424–428.

Pârvulescu L, Schrimpf A, Kozubíková E, Resino SC, Vrålstad T, Petrusek A, Schulz R (2012) Invasive crayfish and crayfish plague on the move: first detection of the plague agent *Aphanomyces astaci* in the Romanian Danube. Diseases of Aquatic Organisms, 98: 85-94.

Persson M, Cerenius L, Söderhäll K (1987) The influence of haemocyte number on the resistance of the freshwater crayfish, *Pacifastacus leniusculus* Dana, to the parasitic fungus *Aphanomyces astaci*. J Fish Dis, 10: 471–477.

Reiland J, Hodge S and Noor MAF (2002) Strong Founder Effect in *Drosophila pseudoobscura* Colonizing New Zealand from North America, J Hered, 93(6): 415-420.

Ryder OA (1986) Species conservation and systematics: the dilemma of subspecies. Trends Ecol Evol*,* 1: 9-10.

Sandodden R and Johnsen SI (2010) Eradication of introduced signal crayfish *Pacifastacus leniusculus* using the pharmaceutical BETAMAX VET®. Aquat Inv, 5: 75–81.

SCBD (Secretariat of the Convention on Biological Diversity) (1992) Convention on Biological Diversity. Available at: www.cbd.int (accessed 29 December 2012)

Scholtz G and Richter S (1995) Phylogenetic systematics of reptantian Decapoda (Malacostraca: Crustacea). Zool J Linn Soc 113: 289– 328.

Schulz R (2000) Status of the noble crayfish *Astacus astacus* (L.) in Germany: monitoring protocol and the use of RAPD markers to assess the genetic structure of populations. Bull Fr Pêche Piscic*.,* 356: 123–138.

Schulz HK and Grandjean F (2005) Phylogeny of European crayfish – improving the taxonomy of European crayfish for a better conservation Bull Fr Pêche Piscic*.* 376-377: 829-83.

Schulz HK, Smietana P and Schulz R (2004) Assessment of DNA variations of the noble crayfish (*Astacus astacus* L.) in Germany and Poland using inter-simple sequence repeats (ISSRs). Bull Fr Pêche Piscic, 372-373: 387–399.

Schulz HK, Smietana P, Maiwald T, Oidtmann B and Schulz R (2006) Case studies on the co-occurrence of *Astacus astacus* (L.) and *Orconectes limosus* (Raf.): snapshots of a slow displacement. Freshw Crayfish 15: 212–219.

Schrimpf A, Dahlem J, Maguire I, Pârvulescu L, Schulz HK, Theissinger K and Schulz R (unpublished) Recolonisation of Europe by noble crayfish (*Astacus astacus*) – natural versus human-mediated processes.

Schrimpf A, Chucholl C, Schmidt T**,** Schulz R (2013) Crayfish plague agent detected in populations of the invasive North American crayfish *Orconectes immunis* (Hagen, 1870) in the Rhine River, Germany. Aquatic Invasions, 8.

Schrimpf A, Maiwald T, Vrålstad T, Schulz HK, Smietána P, Schulz R (in press) Absence of the crayfish plague pathogen (*Aphanomyces astaci*) facilitates coexistence of European and American crayfish in central Europe. Freshwater Biology.

Schrimpf A, Schulz HK, Theissinger K, Pârvulescu L, Schulz R. (2011) First large-scale genetic analysis of the vulnerable noble crayfish *Astacus astacus* reveals low haplotype diversity of central European populations. Knowledge and Management of Aquatic Ecosystems. 401: 35.

Schrimpf A**,** Pârvulescu L, Copila-Ciocianu D, Petrusek A, Schulz R (2012) Crayfish plague pathogen detected in the Danube Delta – a potential threat to freshwater biodiversity in southeastern Europe. Aquatic Invasions, 7(4): 503–510.

Skov C, Aarestrup K, Sivebæk F, Pedersen S, Vrålstad T and Berg S (2011) Non-indigenous signal crayfish *Pacifastacus leniusculus* are now common in Danish streams: preliminary status for national distribution and protective actions. Biol Invasions, 13: 1269–1274.

Skurdal J and Taugbøl T (2002) *Astacus*. In: Holdich DM (ed.), Biology of Freshwater Crayfish, Blackwell Science Ltd, Oxford, United Kingdom, 467–510.

Smietana P, Schulz HK, Keszka S and Schulz R (2006) A proposal for accepting *Pontastacus* as a genus of European crayfish within the family Astacidae based on a revision of the west and east European taxonomic literature. Bull Fr Pêche Piscic, 380-381: 1041-1052.

Söderhäll K and Cerenius L (1992) Crustacean immunity. Annu Rev Fish Dis. 2: 3–23.

Svoboda J, Kozubíková E, Kozák P, Kouba A, Bahadir Koca S, Diler Ö, *et al*. (2012) PCR detection of the crayfish plague pathogen in narrow- clawed crayfish inhabiting Lake Eğirdir in Turkey. Dis of Aquat Organ, 98: 255-259.

Taberlet P, Fumagalli L, Wust-Saucy AG and Cosson JF (1998) Comparative phylogeography and postglacial colonization routes in Europe. Mol Ecol, 7: 453-464.

Taugbøl T (2004) Reintroduction of noble crayfish *Astacus astacus* after crayfish plague in Norway. Bull Fr Peche Piscic, 372-373: 315−328.

Taugbøl T and Peay S (2004) Roundtable Session 3. Reintroduction of native crayfish and habitat restoration. Bull Fr Pêche Piscic, 372-373: 465–471.

Taylor CA (2002) Taxonomy and conservation of native crayfish stocks. In: Holdich DM (ed.), Biology of Freshwater Crayfish, Blackwell Science, Oxford, United Kingdom, 236–257.

Theissinger K, Bálint M, Feldheim K, Haase P, Johannesen J, Laube I and Pauls SU (2012) Glacial survival and postglacial recolonization of an arctic-alpine freshwater insect (*Arcynopteryx dichroa*, Plecoptera, Perlodidae) in Europe. J Biogeogr DOI: doi:10.1111/j.1365-2699.2012.02793.x.

Trontelj P, Machino Y and Sket B (2005) Phylogenetic and phylogeographic relationships in the crayfish genus Austropotamobius inferred from mitochondrial COI gene sequences. Mol Phylogenet Evol, 34: 212–226.

Tuffs S and Oidtmann B (2011) A comparative study of molecular diagnostic methods designed to detect the crayfish plague pathogen, *Aphanomyces astaci*. Vet Microbiol, 153: 343–353.

Viljamaa-Dirks S, Heinikainen S, Nieminen M, Vennerström P and Pelkonen S (2011) Persistent infection by crayfish plague *Aphanomyces astaci* in a noble crayfish population – a case report. B Eur Assoc Fish Pat, 31(5): 182–188.

Vonlanthen P, Excoffier I, Bittner D, Persat H, Neuenschwander S and Largiadèr CR (2007) Genetic analysis of potential postglacial watershed crossings in Central Europe by the bullhead (*Cottus gobio* L.) Mol Ecol 16(21): 4572-4584.

Vrålstad T, Johnsen S, Fristad R, Edsman L and Strand D (2011) Potent infection reservoir of crayfish plague now permanently established in Norway. Dis Aquat Organ, 97: 75-83.

Vrålstad T, Knutsen AK, Tengs T, Holst-Jensen A (2009) A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. Vet Microbiol 137: 146–155.

Weiss S, Persat H, Eppe R, Schlötterer C and Uiblein F (2002) Complex patterns of colonization and refugia revealed for European grayling *Thymallus thymallus*, based on complete sequencing of the mitochondrial DNA control region. Mol Ecol, 11: 1139–1407.

Westman K and Savolainen R (2001) Long term study of competition between two co-occurring crayfish species, the native *Astacus astacus* L. and the introduced *Pacifastacus leniusculus* Dana, in a Finnish lake. Bull Fr Pêche Piscic, 361: 613-627.

Yao Y, Nellaker C and Karlsson H (2006) Evaluation of minor groove binding probe and TaqMan probe PCR assays: Influence of mismatches and template complexity on quantification. Mol Cell Probe, 20: 311–316.

# APPENDIX

## Appendix 1

Schrimpf A, Schulz HK, Theissinger K, Pârvulescu L, Schulz R. (2011) First large-scale genetic analysis of the vulnerable noble crayfish *Astacus astacus* reveals low haplotype diversity of central European populations. Knowledge and Management of Aquatic Ecosystems. 401: 35.

## Appendix 2

Schrimpf A, Dahlem J, Maguire I, Pârvulescu L, Schulz HK, Theissinger K and Schulz R (unpublished) Recolonisation of Europe by noble crayfish (*Astacus astacus*) – natural versus human-mediated processes.

## Appendix 3

Schrimpf A, Chucholl C, Schmidt T**,** Schulz R (2013) Crayfish plague agent detected in populations of the invasive North American crayfish *Orconectes immunis* (Hagen, 1870) in the Rhine River, Germany. Aquatic Invasions, 8.

## Appendix 4

Pârvulescu L, Schrimpf A, Kozubíková E, Resino SC, Vrålstad T, Petrusek A, Schulz R (2012) Invasive crayfish and crayfish plague on the move: first detection of the plague agent *Aphanomyces astaci* in the Romanian Danube. Diseases of Aquatic Organisms, 98: 85-94.

## Appendix 5

Schrimpf A**,** Pârvulescu L, Copila-Ciocianu D, Petrusek A, Schulz R (2012) Crayfish plague pathogen detected in the Danube Delta – a potential threat to freshwater biodiversity in southeastern Europe. Aquatic Invasions, 7(4): 503–510.

## Appendix 6

Schrimpf A, Maiwald T, Vrålstad T, Schulz HK, Smietána P, Schulz R (in press) Absence of the crayfish plague pathogen (*Aphanomyces astaci*) facilitates coexistence of European and American crayfish in central Europe. Freshwater Biology.

Knowledge and Management of Aquatic Ecosystems (2011) **401**: 35.

# *The first large-scale genetic analysis of the vulnerable noble crayfish Astacus astacus reveals low haplotype diversity in central European populations*

**A. Schrimpf[1], H.K. Schulz[1], K. Theissinger[1], L. Pârvulescu[2], R. Schulz[1]**

[1] Institute for Environmental Sciences, University Koblenz-Landau, Fortstrasse 7, 76829 Landau, Germany
[2] Faculty of Chemistry, Biology, Geography, West University of Timisoara, Timisoara, Romania

## ABSTRACT

Major global changes (*e.g.*, human impact or climatic cycles) have a severe impact on the distribution and diversity of species such as the vulnerable European noble crayfish *Astacus astacus*. This is the first large-scale study regarding haplotype diversity of *A. astacus* in central and southeastern Europe. We analyzed a partial sequence of the mitochondrial gene cytochrome oxidase subunit I from 416 specimens of 92 crayfish stocks of three European river basins (Black Sea, North Sea and Baltic Sea). Twenty-two haplotypes were identified, and one common haplotype was found, across the whole study area. We detected differences in the genetic diversity between major river catchments ($\Phi_{ST}$: 0.03481 to 0.20387). The high haplotype diversity ($H_D$ = 0.794±0.024) and high number of private haplotypes suggests a glacial refuge in the Balkan area. The very low haplotype diversity in central Europe ($H_D$ = 0.299 ± 0.038 and $H_D$ = 0.163 ± 0.058) could be a result of human translocation and/or founder effects due to postglacial recolonization. Nevertheless, the high frequency of private haplotypes in all major catchment areas indicates a differentiation of noble crayfish populations throughout Europe despite the extensive human translocation of crayfish. The results of this study support the establishment of conservation management plans for this vulnerable species.

**Key words**: mtCOI sequences • haplotype diversity • human translocation • conservation management • evolutionary significant units (ESU)

## RÉSUMÉ

**Une première analyse génétique à grande échelle des populations vulnérables de l'écrevisse *Astacus astacus* révèle une faible diversité des haplotypes dans les populations du centre de l'Europe**

D'importants changements globaux (par exemple, l'impact humain ou les cycles climatiques) ont un grave impact sur la distribution et la diversité des espèces telle que l'écrevisse à pattes rouges européenne *Astacus astacus*. C'est la première étude à grande échelle en matière de diversité des haplotypes d'*A. astacus* en Europe centrale et du sud-est. Nous avons analysé une séquence partielle du gène mitochondrial de la cytochrome oxydase, sous-unité I de 416 spécimens de 92 stocks d'écrevisses de trois bassins hydrographiques européens (Mer Noire, Mer du Nord et Mer Baltique). Vingt-deux haplotypes ont été identifiés, et un haplotype commun a été trouvé dans toute la zone d'étude. Nous avons détecté des différences dans la diversité génétique entre les principaux bassins versants (ΦST : 0,03481 à 0,20387). La forte diversité des haplotypes (*H*D = 0,794 ± 0,024) et le nombre élevé d'haplotypes suggèrent un refuge glaciaire dans la région des Balkans. La très faible diversité des haplotypes en Europe centrale (0,299 ± *H*D = 0,038 et 0,163 ± *H*D = 0,058) pourrait être une conséquence de la translocation de l'homme et / ou des effets fondateurs en raison de la re-colonisation postglaciaire. Néanmoins, la fréquence élevée des haplotypes particuliers dans tous les grands bassins versants indique une différenciation des populations d'écrevisses à pattes rouges à travers l'Europe, malgré les translocations importantes de l'écrevisse. Les résultats de cette étude viennent appuyer la mise en place de plans de conservation pour cette espèce vulnérable.

**Mots-clés:** séquences mtCOI • diversité des haplotypes  translocation • gestion de la conservation • evolutionary significant units (ESU)

## INTRODUCTION

Genetic diversity among and within species is the basis of evolution and is important for the resilience of native stocks to future changes in the environment (Östergren, 2006). The convention on biological diversity (CBD) highlighted the importance of genetic diversity within species as one of the three levels of biological diversity (CBD, adopted 1992). Therefore, the identification of genetically differentiated populations is crucial for the conservation and management of a species or the regional strains within a species. The guidelines for the protection of threatened species recommend the identification of evolutionary significant units (ESU, Ryder, 1986). An ESU may be defined as a population or group of populations that is sufficiently differentiated and requires a separate management or conservation strategy (Moritz, 1994). Defining management units can aid in selecting a management program among the various populations so that the greatest overall diversity is sustained by the conservation plan (Crozier, 1997). Nevertheless, conservation managers typically do not use genetic evidence (*i.e.*, the identification of ESUs) to support their adopted measures (Pullin *et al.*, 2004). The IUCN red list of threatened species cites *Astacus astacus* as vulnerable with a decreasing population status (Edsman *et al.*, 2010). *A. astacus* is widely distributed across Europe. Its range extends from Russia and the Ukraine in the east, Scandinavia in the north, Greece in the south, and the United Kingdom and France in the west (Edsman *et al.*, 2010). Based on morphological criteria, different and partly contradictory classifications of the noble crayfish subspecies can be found in the literature (see Smietana *et al.*, 2006 for a review). Here, we focus on the taxonomic studies of Karaman (1962) and Albrecht (1983). They described three subspecies with distinct geographical distributions: (1) *A. a. colchicus* is endemic to an isolated area in the Ponto-Caspian region of the upper Rion (Caucasus). (2) *A. a. balcanicus* inhabits the western Balkans in the drainage system of the Vardar river in Macedonia and Greece, as well as Lake Ohrid. (3) *A. a. astacus* inhabits the Danube river and its tributaries, as well as the rivers that open out into the North and Baltic Seas. The fragmented nature of *A. astacus*' typical freshwater habitats (streams, rivers and lakes with sufficient water quality and an excess of shelter) coupled with a low migratory potential

reduces the possibility of reestablishing local populations that have gone extinct (Abrahamsson, 1971; Strayer and Dudgeon, 2010). In addition to environmental contributions to the decreasing number of stocks (*i.e.*, water pollution or channelization of streams), the oomycete *Aphanomyces astaci* is decreasing *A. astacus*' numbers (Schikora, 1906). This invasive pathogen was brought to Europe *via* American crayfish species (*e.g.*, *Orconectes limosus*) in the mid-nineteenth century (Alderman *et al.*, 1990) and caused massive die-offs in native crayfish stocks (Alderman, 1996). During the Middle Ages, the noble crayfish became a commercial object throughout Europe, and it has been sold as a delicacy ever since (Schulz *et al.*, 2004). The translocation of individuals and whole stocks (Skurdal and Taugbøl, 2002), sometimes over large distances (Koutrakis *et al.*, 2007), resulted in the foundation of new stocks (Albrecht, 1983) and in the mixing of indigenous and non-indigenous populations. Over the last decades, the number of stocking events that disregard the genetic structure within and between populations (Souty-Grosset and Reynolds, 2009) and cross-basin translocations in response to rapidly declining stocks have led to a contamination of local stocks (Largiadèr *et al.*, 2000). This has led to repeated calls for modern conservation programs that consider the genetic origin of the stocking material (*e.g.*, Schulz *et al.*, 2004; Bertocchi *et al.*, 2008; Souty-Grosset and Reynolds, 2009; Strayer and Dudgeon, 2010). Previous studies on noble crayfish taxonomy and biogeography are based solely on morphological characteristics (Bott, 1950, 1972; Karaman, 1962, 1963; Albrecht, 1983), while molecular studies have only focused on restricted geographical areas (Agerberg, 1990; Fevolden *et al.*, 1994; Schulz, 2000; Edsman *et al.*, 2002; Schulz *et al.*, 2004; Alaranta *et al.*, 2006). This study is the first molecular-based study of *A. astacus* covering a large portion of its distribution range, including river catchments of the North and Baltic Seas in central Europe and the Black Sea in southeastern Europe. The main aim of this study was to determine the degree of genetic structure in noble crayfish populations across three major catchments areas. In particular, we focused on asking to what extent this structure is the consequence of natural migration due to past climatic fluctuations during the Pleistocene or recent man-induced translocations. In the latter case, we would expect a lower genetic structure due

to shared haplotypes between geographically disconnected populations. An anthropogenic influence could have caused a homogenization of haplotypes throughout Europe. The identification of inter-basin diversity could result in the conservation of catchment-specific gene pools (*i.e.*, the identification of ESUs) to protect the present-day genetic diversity (Weiss *et al.*, 2002). Finally, the results of this study are discussed in the context of conservation management plans for this vulnerable species.

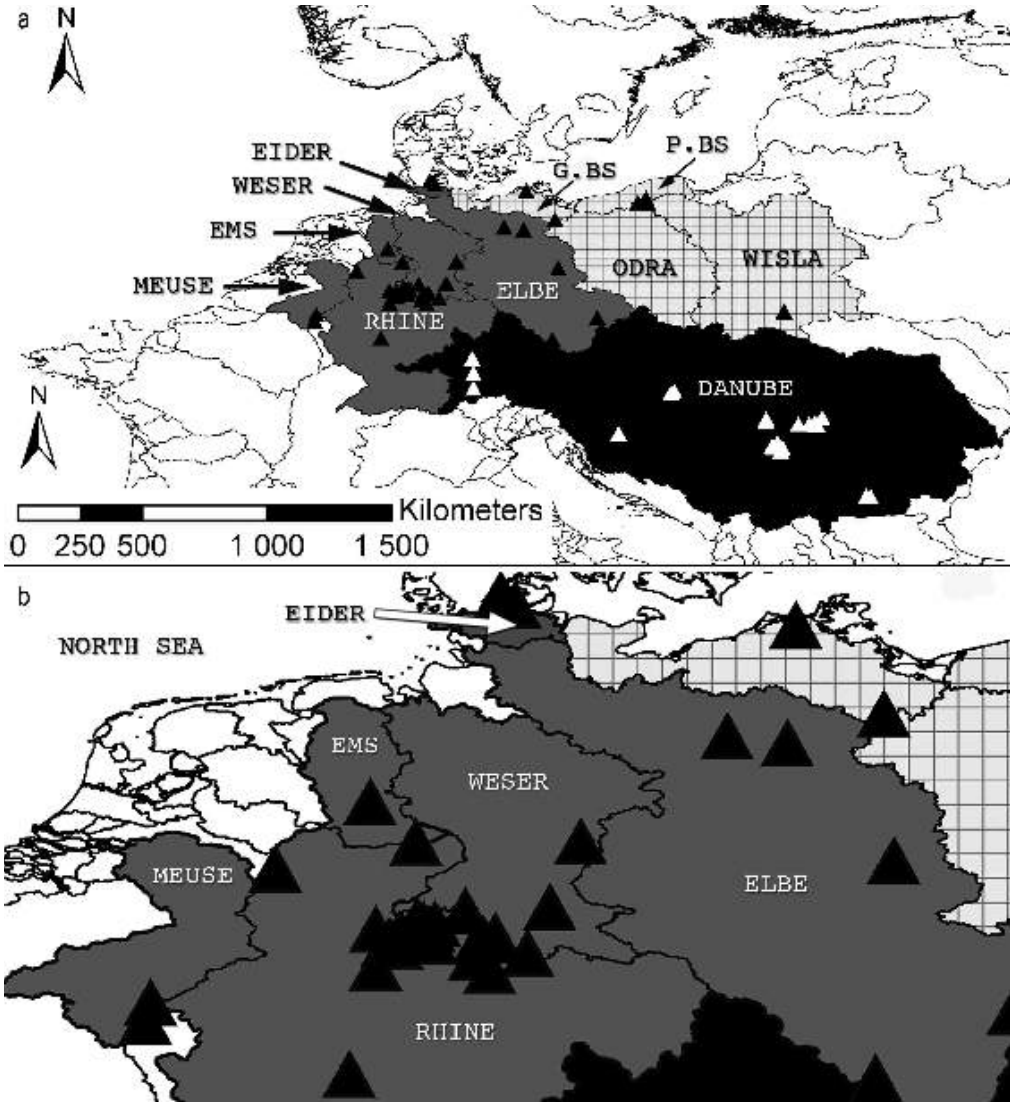


**Figure 1** (a) Study area with sampling sites. Major catchment areas of European rivers are indicated by a checked pattern for the Baltic Sea catchment area; by dark grey for the North Sea catchment area; and by black for the Black Sea catchment area. G. BS = German Baltic Sea estuary, P. BS = Polish Baltic Sea estuary. (b) Larger scale map of the North Sea major catchment.

## MATERIALS AND METHODS

*Sampling*

A total of 416 crayfish specimens from 92 sampling locations (Figure 1,Tables I and II) were collected either by hand or with traps within catchments in the North, Baltic, and Black Seas. Immediately after the lower part of one pereopod (propodus and dactylus) was taken, specimens were released at the place where they were caught. Appendages usually regenerate after a few molts. The samples were stored in 96% ethanol until DNA extraction. DNA was extracted from the muscle tissue using a standardized protocol ('rapid isolation of Mammalian DNA', Sambrook and Russel, 2001).

*Laboratory procedures*

A 350 base pair (bp) fragment of the mitochondrial cytochrome oxidase subunit I (COI) was sequenced using the primer pair ASTCOI (forward primer: 5'-GCGGGGATAGTAGGAACCTC-3'; reverse primer: 5'-ATTTACCGCCCCTAAAATCG-3'; Schrimpf and Schulz, in prep.). Polymerase chain reactions (PCR) were performed in a total volume of 25 µL containing 0.625u GoTaq DNA Polymerase (Promega, Mannheim, Germany), 1× Colorless GoTaq® Flexi Buffer (Promega, Mannheim, Germany), 2 mM $MgCl_2$ (Promega, Mannheim, Germany), 0.24 mM of each dNTP (Fermentas, St. Leon-Rot, Germany), 0.2 µM of each primer and 20 ng of template DNA. The PCR reaction was performed using a Primus 96 Cycler (Peqlab Biotechnologie GmbH, Erlangen, Germany) under the following conditions: an initial 2 min step at 95 °C, followed by 35 cycles with 45 s at 95 °C, 45 s at 50 °C, 1 min at 72 °C, and a final extension of 5 min at 72 °C. PCR products were stored at 10 °C. PCR products were partly sequenced on a 3730 DNA Analyzer eight capillary sequencer (Applied Biosystems, MA, USA) by the company SeqIT (Kaiserslautern, Germany). The remaining PCR product was purified with the AMPure XP system (Beckman Coulter, Krefeld, Germany). The fragments were amplified in a sequencing-reaction using the primer ASTCOIF and the CEQ DTCS-Quick Start kit (Beckman Coulter, Krefeld, Germany). After ethanol precipitation, sequencing was performed on a Beckman Coulter CEQ 8000 eight capillary sequencer. Each

template was sequenced in the forward direction, and 50% of samples were randomly chosen for sequencing in the reverse direction. The sequences were edited and aligned with the Sequencher 3.0 software (gene codes corporation, Ann Arbor, Michigan). The sequences were checked manually for base pair ambiguities, nuclear copies of mitochondrial derived genes, stop codons, and high levels of divergence, as recommended by Buhay (2009). All haplotypes were submitted to GenBank.

*Statistical Analyses*

A median joining (MJ) network (Bandelt *et al.*, 1999) was constructed using the software network 4.510 (www.Fluxus-Engineering.com, 2009) to identify haplotypes and to determine the phylogenetic relationships between haplotypes, which allows us to identify geographically localized haplogroups. The network was also calculated with TCS 1.21 (Clement *et al.*, 2000) software to confirm the shape of the network. Haplotype diversity ($H_D$) and the number of polymorphic sites ($S$) per river catchment were calculated using the software ARLEQUIN 3.5.1 (Excoffier *et al.*, 2005). The calculation of haplotype diversity is based on the frequency of a haplotype and the sample size (Nei, 1987). To detect genetic differentiation among major catchment areas, an analysis of the molecular variance (AMOVA, Excoffier *et al.*, 1992), based on three hierarchical groups corresponding to the major catchment areas, and the pairwise $\Phi_{ST}$-values (Weir and Cockerham, 1984) were calculated using the program ARLEQUIN 3.5.1. We further examined population genetic differentiation using exact tests of population differentiation (ETPD, Raymond and Rousset, 1995), as implemented in ARLEQUIN 3.5.1. In addition, $H_D$, $S$, and pairwise $\Phi_{ST}$-values (Weir and Cockerham, 1984) were calculated for sampling sites in the North Sea catchment area. Here, the Ems population has been excluded from the calculation of the pairwise $\Phi_{ST}$-values due to the low sample size ($N = 5$).

**RESULTS**

COI sequences were analyzed for 416 specimens from 96 sample sites across three major catchment areas in Europe (Tables I and II, Figure 1). There were 20 variable sites within the 350-bp fragment (0.06%). Only four of these

substitutions resulted in an amino acid change in four haplotypes (H04, H10, H21). In total, 23 unique haplotypes were detected (GenBank accession numbers: JN254659-JN254681), 17 of which were endemic to a specific river catchment. The overall haplotype diversity was 41.9%. The median joining (MJ) network is shown in Figure 2.The shape was confirmed with the TCS software. The greatest observed distance between haplotypes was 9 bp. For the Black Sea catchment, 10 out of 16 haplotypes were endemic to this region. Of the 11 North Sea haplotypes, five were endemic to the catchment area, and two out of three haplotypes were exclusively found in the rivers discharging into the Baltic Sea. Five haplotypes (H05, H12, H17, H18, H20) were shared between populations in the Black and North Seas. One haplotype (H01), representing the center of the MJ network, was found frequently in all regions, resulting in a star-like topology of the network. A list of all haplotypes for each sampling site is given in Table II.

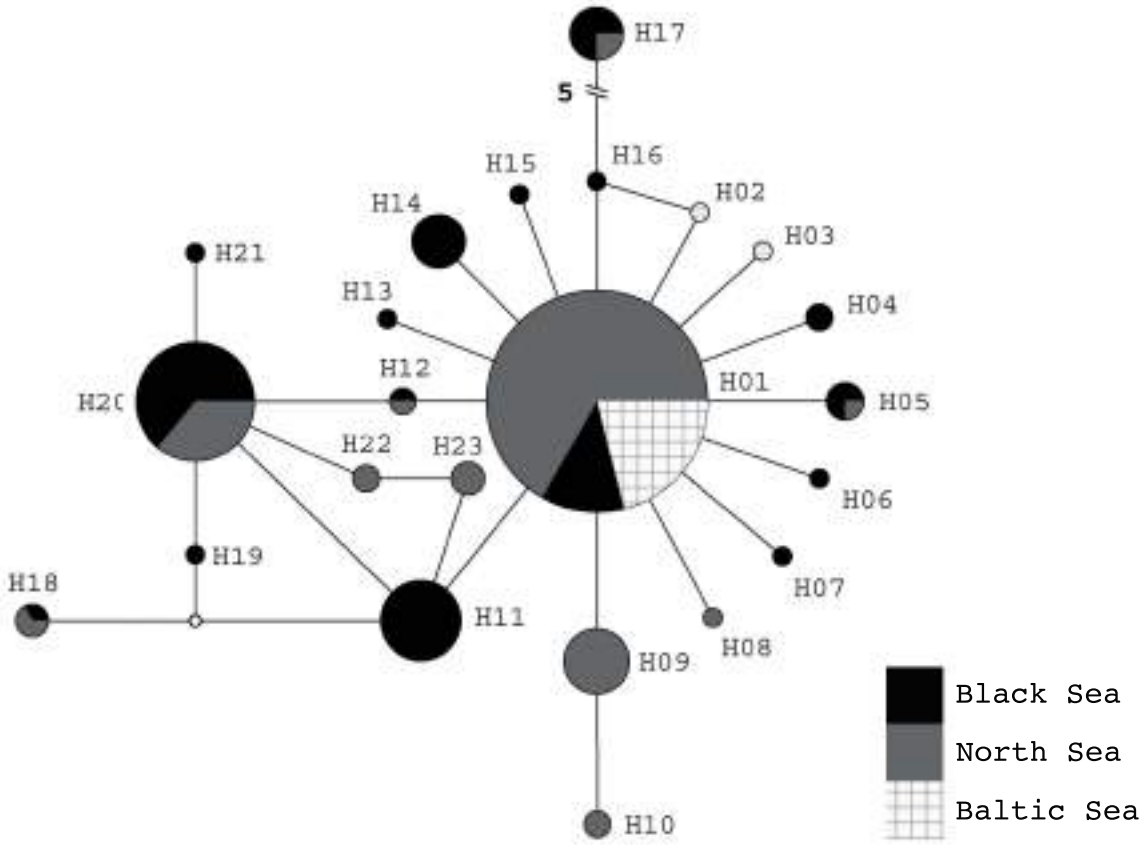


**Figure 2** Median joining network of COI haplotypes (350 bp) from 416 individuals of *Astacus astacus*.The size of the circles is proportional to the frequency of the haplotypes. Median vectors are indicated as white dots. The number of base pair (bp) changes are given; no number = 1 bp change.

**Table I** Locality information of analyzed *Astacus astacus* populations. The originating countries and river catchments, the number of populations (Pop) and the number of sequenced individuals (N), the number of haplotypes (HN), the haplotype diversity (HD) and the number of polymorphic sites (S) are given. Country Codes: AUT = Austria; BEL = Belgium: BUL = Bulgaria; CZE = Czech Republic; GER = Germany; CRO = Croatia; HUN = Hungary; POL = Poland; ROU = Romania.

| Major catchment area | Countries | River catchments | Pop | N | $H_N$ | $H_D$ | S |
|---|---|---|---|---|---|---|---|
| Black Sea | AUT, BUL, CRO, GER, HUN, ROU | Danube | 30 | 106 | 16 | 0.794±0.024 | 17 |
| North Sea | CZE, GER, BEL | Eider, Elbe, Rhine, Meuse, Weser | 50 | 241 | 11 | 0.299±0.038 | 14 |
| Baltic Sea | GER, POL | Uecker, Odra, Wisla, Wiprza | 16 | 69 | 3 | 0.163±0.058 | 2 |

$H_D$-values (Table I) were lower for the rivers in central Europe (North Sea: $H_D$ = 0.299 ± 0.038 and Baltic Sea: $H_D$ = 0.163 ± 0.058) than for the Black Sea catchment ($H_D$ = 0.794 ± 0.024). Pairwise comparisons between river catchments revealed the lowest ΦST-value between the North and the Baltic Seas (ΦST = 0.035), and the highest value between the Black and Baltic Seas (ΦST = 0.204, Table V). The ΦST-value between the North Sea and the Black Sea was 0.161. All values were highly significant ($p$ < 0.001). The results of the AMOVA based on the three hierarchical groups (the major catchment areas) are given in Table III and reveal highly significant genetic differentiation between the major catchment areas (ΦST = 0.290, $p$ < 0.001) and among river catchments within the major catchment areas ($F$SC = 0.252, $p$ < 0.001). $H_D$-values for river catchments that open into the North Sea ranged between 0.000 (Weser) and 0.400 (Ems), although these minimum and maximum values could be due to low sample sizes (Weser: $N$ = 12; Ems: $N$ = 5; see Table IV). $H$D for the Rhine ($N$ = 161) was 0.227. Pairwise ΦST-values for the North Sea major catchment area (Table VI) revealed very low genetic differentiation between sampled rivers. However, the Eider exhibited a high differentiation compared to all of the other rivers of the North Sea catchment area (ΦST-value between 0.575 and 0.876; $p$ < 0.001).

**Table II** Sampling locations and haplotypes of analyzed *Astacus astacus* and the number of the most common haplotype (H01) and the number of all other haplotypes. WIE = Wieprza, WIS = Wisla, DA = Danube, RH = Rhine, G. BS = German Baltic Sea estuary, P. BS = Polish Baltic Sea estuary, BAS = Baltic Sea, BS = Black Sea, NS = North Sea. n.a. = not available. For country codes, see Table I.

| Region | [N°] | [E°] | Country | River district | Estuary | N | H01 | Haplotype |
|---|---|---|---|---|---|---|---|---|
| Barthe | 54.333 | 12.833 | GER | G. BS | BAS | 5 | | H02(5) |
| Tonkuhle | 54.333 | 12.833 | GER | G. BS | BAS | 3 | 3 | |
| Lake close to Miastko | 54.017 | 16.983 | PO | P. BS | BAS | 10 | 10 | |
| Lake close to Miastko | 54.017 | 16.983 | PO | P. BS | BAS | 7 | 7 | |
| Lake close to Miastko | 54.017 | 16.983 | PO | P. BS | BAS | 8 | 8 | |
| Rakowe Duze | 53.915 | 16.796 | PO | Odra | BAS | 5 | 5 | |
| Koppelsee | 53.307 | 13.855 | GER | Uecker | BAS | 3 | 3 | |
| Seki | 54.065 | 17.095 | PO | WIE | BAS | 5 | 5 | |
| Bez Nazwu | 54.067 | 17.083 | PO | WIE | BAS | 4 | 4 | |
| Rosko | 53.903 | 17.143 | PO | WIS | BAS | 5 | 4 | H03(1) |
| Czarne | 50.038 | 21.993 | PO | WIS | BAS | 6 | 6 | |
| Tomczyna | 50.036 | 22.004 | PO | WIS | BAS | 8 | 8 | |
| Freundsheimer Weiher | 47.304 | 10.954 | A | DA | BS | 3 | 2 | H18(1) |
| Razdvec | 43.463 | 24.908 | BU | DA | BS | 6 | 4 | H13(1)<br>H15(1) |
| Beli Osam | 42.858 | 24.655 | BU | DA | BS | 3 | | H14(3) |
| Gorna Trape | 43.444 | 25.073 | BU | DA | BS | 6 | 1 | H14(4)<br>H16(1) |
| Farm Augsburg | 48.359 | 10.906 | GER | DA | BS | 14 | 9 | H05(2)<br>H06(1)<br>H20(2) |
| Wielenbach | 47.841 | 10.952 | GER | DA | BS | 2 | 2 | |
| Vukovina | 45.693 | 16.109 | HR | DA | BS | 5 | | H17(5) |
| Kádárta | 47.120 | 17.962 | HU | DA | BS | 2 | 2 | |
| Pét | 47.184 | 18.096 | HU | DA | BS | 1 | | H07(1) |
| Clocotici | 45.241 | 21.838 | RO | DA | BS | 8 | | H20(7)<br>H12(1) |
| Bradisoru de Jos | 45.104 | 21.768 | RO | DA | BS | 1 | | H11(1) |
| Carasova | 45.174 | 21.952 | RO | DA | BS | 1 | | H11(1) |
| Carasova | 45.176 | 21.943 | RO | DA | BS | 1 | | H11(1) |
| Garliste | 45.152 | 21.873 | RO | DA | BS | 5 | | H11(5) |
| Anina | 45.059 | 21.887 | RO | DA | BS | 2 | 2 | |
| Ocna de Fier | 45.354 | 21.759 | RO | DA | BS | 4 | 1 | H11(3) |
| Forotic | 45.251 | 21.574 | RO | DA | BS | 6 | | H11(6) |
| Carnecea | 45.206 | 21.636 | RO | DA | BS | 1 | | H11(1) |
| Hartagani | 46.06 | 22.925 | RO | DA | BS | 1 | | H21(1) |
| Balsa | 46.019 | 23.12 | RO | DA | BS | 1 | 1 | |
| Baita | 46.033 | 22.884 | RO | DA | BS | 2 | 2 | |
| Bacaia | 46.01 | 23.171 | RO | DA | BS | 2 | 2 | |
| Balsa | 46.04 | 23.07 | RO | DA | BS | 2 | 1 | H20(1) |

| Region | [N°] | [E°] | Country | River district | Estuary | N | H01 | Haplotype |
|---|---|---|---|---|---|---|---|---|
| Tamasesti | 46.022 | 22.507 | RO | DA | BS | 3 | 3 | |
| Almasu Mic de Munte | 46.052 | 23.072 | RO | DA | BS | 3 | 2 | H19(1) |
| Cladova | 46.151 | 21.357 | RO | DA | BS | 4 | *2* | H04(2) |
| Araneag | 46.21 | 21.357 | RO | DA | BS | 2 | 1 | H20(1) |
| Nadas | 46.206 | 21.359 | RO | DA | BS | 14 | | H12(1)<br>H20(12)<br>H11(1) |
| Conop | 46.122 | 21.359 | RO | DA | BS | 1 | | H20(1) |
| Farm Oeversee | 54.688 | 9.422 | GER | Eider | NS | 5 | | H09(4)<br>H10(1) |
| Langsee | 54.575 | 9.593 | GER | Eider | NS | 9 | | H09(8)<br>H10(1) |
| Svetlohor | 48.977 | 13.736 | CZ | Elbe | NS | 7 | 7 | |
| U sudu | 49.822 | 15.358 | CZ | Elbe | NS | 6 | 4 | H18(2) |
| Kramata | 49.043 | 13.758 | CZ | Elbe | NS | 7 | 7 | |
| Stepenitz | 53.037 | 12.042 | GER | Elbe | NS | 5 | 5 | |
| Schwarze Elster | 51.582 | 13.97 | GER | Elbe | NS | 6 | 6 | |
| Jäglitz | 52.96 | 12.741 | GER | Elbe | NS | 7 | 5 | H17(2) |
| Gut Rietberg | 51.802 | 8.435 | GER | Ems | NS | 5 | 4 | H20(1) |
| Florenville | 49.700 | 5.307 | BE | Meuse | NS | 5 | 5 | |
| Libramont | 49.927 | 5.371 | BE | Meuse | NS | 4 | 4 | |
| n.a. | n.a. | n.a. | BE | Meuse | NS | 2 | 1 | H12(1) |
| Fischbach | 49.087 | 7.674 | GER | RH | NS | 6 | 3 | H23(3) |
| Klausbach | 52.272 | 7.910 | GER | RH | NS | 7 | 3 | H20(4) |
| Mahlscheid | 50.778 | 7.972 | GER | RH | NS | 8 | 8 | |
| Zoo Zajak, Petstore | 51.488 | 6.810 | GER | RH | NS | 4 | *4* | |
| Aar | 50.675 | 8.487 | GER | RH | NS | 5 | 5 | |
| Allna | 50.791 | 8.591 | GER | RH | NS | 4 | 4 | |
| Ambach | 50.702 | 8.275 | GER | RH | NS | 5 | 5 | |
| Mühlgraben Caldern | 50.842 | 8.658 | GER | RH | NS | 3 | 2 | H05(1) |
| Dautphe | 50.838 | 8.525 | GER | RH | NS | 3 | 3 | |
| TA Donsbach | 50.708 | 8.242 | GER | RH | NS | 5 | 5 | |
| Eichelbach | 50.459 | 9.123 | GER | RH | NS | 5 | 5 | |
| Fohnbach | 50.636 | 8.622 | GER | RH | NS | 5 | 5 | |
| Gansbach | 50.804 | 8.409 | GER | RH | NS | 5 | 5 | |
| TA Geierstein/ Roth | 50.641 | 8.226 | GER | RH | NS | 5 | 5 | |
| Giebelsbach | 50.997 | 9.012 | GER | RH | NS | 1 | 1 | |
| Kallenbach | 50.582 | 8.226 | GER | RH | NS | 10 | 10 | |
| Lasterbach/ TA Mabüll | 50.591 | 8.142 | GER | RH | NS | 5 | 5 | |
| Merzkrebse | 50.336 | 7.976 | GER | RH | NS | 9 | 9 | |
| Meerbach | 50.706 | 8.482 | GER | RH | NS | 1 | 1 | |
| Madenmühlen | 50.624 | 8.143 | GER | RH | NS | 5 | 5 | |
| Mademühlen 2 | 50.624 | 8.143 | GER | RH | NS | 2 | 2 | |
| Nanzenbach | 50.780 | 8.489 | GER | RH | NS | 1 | 1 | |
| Perf | 50.873 | 8.459 | GER | RH | NS | 5 | 5 | |
| TA Hartmann/ Rehbach | 50.624 | 8.120 | GER | RH | NS | 5 | 5 | |

| Region | [N°] | [E°] | Country | River district | Estuary | N | H01 | Haplotype |
|---|---|---|---|---|---|---|---|---|
| Salzbach | 50.753 | 8.539 | GER | RH | NS | 5 | 5 | |
| TA bei Spielberg | 50.304 | 9.298 | GER | RH | NS | 5 | 2 | H20(3) |
| Steinbruch Rot/ Schönbach | 50.654 | 9.219 | GER | RH | NS | 5 | 5 | |
| Stippbach | 50.704 | 9.359 | GER | RH | NS | 5 | 5 | |
| Pollichia Woog | 49.103 | 7.719 | GER | RH | NS | 2 | | H20(2) |
| Pollichia Woog2 | 49.103 | 7.719 | GER | RH | NS | 2 | 1 | H20(1) |
| Schlettenbachtal, Fischbach | 49.088 | 7.711 | GER | RH | NS | 1 | | H20(1) |
| | | | | | | | | H08(1) |
| Wolfsägertal | 49.106 | 7.701 | GER | RH | NS | 7 | 2 | H20(2) |
| | | | | | | | | H22(2) |
| Meisertalweiher | 49.335 | 7.769 | GER | RH | NS | 5 | 5 | |
| Waldteich Irrschelde | 50.763 | 8.399 | GER | RH | NS | 5 | 5 | |
| Waldteich bei Wallenfels | 50.774 | 8.438 | GER | RH | NS | 5 | 5 | |
| Clausthal-Zellerfeld* | 51.809 | 10.352 | GER | Weser | NS | 1 | 1 | |
| Breitweiher/Rhön | 50.507 | 9.730 | GER | Weser | NS | 2 | 2 | |
| Ocherbach | 50.673 | 9.185 | GER | Weser | NS | 5 | 5 | |
| Urff | 51.041 | 9.991 | GER | Weser | NS | 4 | 4 | |

* This sequence was obtained from a preserved specimen of the Senckenberg Museum in Frankfurt (SMF 13095).

**Table III** Results of the analysis of molecular variance (AMOVA). Shown are the percentage of the total variance (% Var), fixation indices (F) and their significance (***: $p < 0.001$)based on 1000 random permutations.

| Variance components | d.f. | % Var | F-statistics |
|---|---|---|---|
| Among major catchment areas | 2 | 5.06 | $\Phi_{CT}$=0.051 |
| Among river catchments within major catchment areas | 9 | 23.92 | $\Phi_{SC}$=0.252*** |
| Within river catchments | 406 | 71.02 | $\Phi_{ST}$=0.290*** |

**Table IV** Diversity values for the North Sea sampling area. The number of sequenced individuals (N), the number of haplotypes ($H_N$),the haplotype diversity ($H_D$) and the number of polymorphic sites (S) for seven rivers flowing into the North Sea are given.

| River catchment | N | $H_N$ | $H_D$ | S |
|---|---|---|---|---|
| Elbe | 38 | 3 | 0.199±0.084 | 8 |
| Ems | 5 | 2 | 0.400±0.237 | 2 |
| Meuse | 11 | 2 | 0.182±0.144 | 1 |
| Rhine | 161 | 6 | 0.227±0.043 | 5 |
| Weser | 12 | 1 | 0.000±0.000 | 0 |
| Eider | 14 | 2 | 0.264±0.136 | 1 |

**Table V** Pairwise $\sqrt{}_{ST}$-values between the three major catchment areas and their significance (*: $p < 0.05$, ***: $p < 0.001$).

| | **Black Sea** | **North Sea** | **Baltic Sea** |
|---|---|---|---|
| Black Sea | 0 | | |
| North Sea | 0.16131*** | 0 | |
| Baltic Sea | 0.20387*** | 0.03481*** | 0 |

**Table VI** Pairwise $\sqrt{}_{ST}$-values between five rivers flowing into the North Sea and their significance (*: $p < 0.05$, ***: $p < 0.001$).

| | **Elbe** | **Meuse** | **Rhine** | **Weser** | **Eider** |
|---|---|---|---|---|---|
| Elbe | 0 | | | | |
| Meuse | -0.011 | 0 | | | |
| Rhine | 0.042* | -0.018 | 0 | | |
| Weser | -0.016 | 0.008 | 0.007 | 0 | |
| Eider | 0.575*** | 0.816*** | 0.699*** | 0.876*** | 0 |

## DISCUSSION

This is the first large-scale molecular study of *Astacus astacus*. The main aim was to determine the degree and origin (natural migration versus artificial translocation) of the genetic structure in noble crayfish populations across three major catchment areas. We detected very low haplotype diversities in noble crayfish populations in the North Sea and Baltic Sea catchments, with the exception of the Eider at the very northern end of Germany. The observed significant structure ($\Phi_{ST}$-values between 0.575 and 0.876) between the Eider and all other North Sea river catchments may be explained by an isolation of the Eider population. The Eider exhibits an east to west current, which is in contrast to the primarily south-north running rivers of southern Germany. This might result in a disconnection of the Eider from the other southern river systems, where the catchment areas of streams partly overlap and gene flow seems more likely. As a peripheral source with restricted gene flow, the Eider population contributes to the overall genetic diversity within *A. astacus*. To discover the origin of the private haplotypes solely found within this river catchment (indigenous or non-indigenous), additional populations nearby should be analyzed. Noble crayfish populations within the central European major catchment areas are significantly less differentiated than within the Black Sea catchment (Table I). A high haplotype

diversity and a high number of private haplotypes were detected for the Black Sea populations, suggesting a glacial refuge of the noble crayfish in the Balkan area. During the Pleistocene, severe climatic fluctuations in central Europe resulted in repeated range expansions and regressions of many taxa. The distribution of freshwater species was especially influenced by the fluctuating glaciers and varying water levels, which resulted in landscape changes and modified river systems (Hänfling *et al.*, 2009). The recolonization of previously glaciated habitats (northern Germany) could have caused successive bottlenecks (Taberlet *et al.*, 1998), which could explain the loss of genetic diversity and the observed dominance of haplotype H01 in the North Sea and Baltic Sea major catchments. A decrease in genetic diversity from southern to northern populations has been observed for many freshwater species, for example, the freshwater fish *Vimba vimba* (Hänfling *et al.*, 2009) or the mayfly *Ameletus inopinatus* (Taubmann *et al.*, 2011). The prevalence of haplotype H01 in the entire study area (Figure 1) may indicate a recolonization event from the Balkan area to central Europe. The fact that the North Sea and Baltic Sea major catchments only share one haplotype and show some degree of differentiation (Tables II and V) maybe interpreted as a result of two routes of recolonization from a common refuge. One migratory pathway into central Europe may have been the Danube drainage system after deglaciation, as presumed by Schulz and Grandjean (2005). The Danube represents a widely used corridor of postglacial expansion into central Europe for many freshwater species for which the possibility of a large-scale terrestrial dispersal has been excluded (*e.g.*, Durand *et al.*, 1999; Weiss *et al.*, 2002; Hänfling *et al.*, 2009). A second recolonization route, which would explain the divergence of the North Sea and Baltic Sea major catchments, cannot be confirmed due to the lack of sample sites from eastern Europe. The occurrence of noble crayfish in the Baltic Sea major catchment may also be the result of human translocation, although no evidence exists to support this theory.

Our study indicates that the original distribution pattern of the noble crayfish has changed significantly due to anthropogenic influence (Skurdal and Taugbøl, 2002), resulting in a mixture of indigenous and non-indigenous populations (Largiadèr *et al.*, 2000). Endemic haplotypes of the North Sea and Baltic Sea

catchments could be the result of artificial stocking, or using stock from a river catchment not included in our study (*e.g.*, from Russia). However, presumably no stocking of native crayfish has been conducted in the Balkan States and the Ponto-Caspian region (Albrecht, 1983). We can therefore assume a natural origin of the studied populations from the Balkans (Maguire, 2009). The high frequency of private haplotypes in all major catchment areas, as well as the relatively high genetic differentiation between the catchment areas (ΦST-values between 0.035 and 0.204), indicates a differentiation of populations throughout Europe despite the extensive human translocation of noble crayfish. Nevertheless, the artificial translocation of individuals can be observed in the disjunct distribution of some shared haplotypes between different drainage areas (Figure 1). Haplotype H20 could be an example of artificial distribution across central Europe. This haplotype was detected in the lower section of the Danube (Black Sea catchment), in the river catchments of the Rhine and Ems, and in individuals from a crayfish farm in Augsburg, Southern Germany (Table VII). This farm was founded by mixing four donor populations of the upper section of the Danube catchment area (Keller, pers. com.). Because restocking programs have been frequently conducted throughout Germany over the last few decades, in particular using stocking materials from the above-mentioned farm in Augsburg (Keller, 1999; Souty-Grosset and Reynolds, 2009), it is conceivable that specimens carrying haplotype H20 may originate from the Danube area and were artificially spread over central Europe. Human impact is also assumed to affect the (haplotype-) distribution of other European crayfish species, such as *Austropotamobius pallipes* (*e.g.*, Grandjean *et al.*, 2001; Diéguez-Uribeondo *et al.*, 2008) or *Austropotamobius italicus* (Cataudella *et al.*, 2010). Until today, the natural migratory routes of these species could not be fully inferred. However, the authors revealed geographical differences in the haplotype distribution and diversity, which may influence the conservation management strategies for these species.

With regard to *A. astacus*, the high frequency (74%) of private haplotypes in the Black Sea catchment highlights the importance of the Balkans as a reservoir for intraspecific genetic diversity. In addition to the topographical complexity, which allows for the isolation of single populations on a small geographical scale,

the Balkans were relatively unaffected by geological events during the glacial cycles. Thus, biodiversity is more conserved in the Balkans compared to central Europe for many species (Trontelj *et al.*, 2005; Hänfling *et al.*, 2009). Therefore, we strongly suggest a definition of several distinct ESUs along the Black Sea major catchment area. To define such ESUs, more populations of the Black Sea catchment area have to be analyzed because there might be much higher levels of genetic diversity and more detectable, endemic haplotyes to be found, for example, in the Upper Kolpa drainage similar to the recently detected distinct clades of *Austropotamobius torrentium* (Trontelj *et al.*, 2005). So far, we suggest avoiding the intermixing of noble crayfish populations between the tributaries of the Danube. This is especially important for countries that plan to found crayfish farms for potential future restocking programs, *e.g.*, in Bulgaria (Zaikov and Hubenova, 2007). We strongly emphasize that haplotype identification of the donor population should be conducted before a breeding stock is used for restocking. Additionally, the waters to be restocked should be carefully selected to avoid the contamination of local stocks. If restocking programs continue to translocate individuals with no regard for their population's genetic structure, the natural genetic make-up will further dissolve (Souty-Grosset *et al.*, 2003), which is accompanied by a reduction in intraspecific diversity. Conservation strategies, therefore, need to manage populations of *A. astacus* as distinct ESUs and give the highest priority to the populations with high genetic diversity and unique haplotypes. Mitochondrial DNA is a widely used marker to reconstruct the phylogeographic history of species. Here, we show that the analysis of partial COI-sequences helps to understand the genetic structure of noble crayfish. However, the variation in noble crayfish COI-sequences is relatively low and the resolution is limited. Additional DNA-sequences should be included in the analysis to increase the resolution. Finally, a microsatellite analysis should be conducted to estimate genetic diversity within populations to give special attention to diverse populations and to further identify artificially stocked and naturally dispersed populations. However, as long as we cannot reject the hypothesis that private central European haplotypes developed due to local adaption, those populations that harbor endemic haplotypes are suggested to be candidates for special management plans to prevent the loss of unique haplotypes and to protect overall

genetic diversity. In conclusion, the results of this study suggest a glacial refuge in the Balkan area and a postglacial re-colonization of central Europe. Despite human translocations, which were revealed by the disjunct distribution of some haplotypes, a differentiation of noble crayfish populations in all major catchment areas supports the establishment of distinct ESUs to protect its present-day genetic diversity in Europe.

## ACKNOWLEDGEMENTS

We thank the following colleagues for providing samples: Erik Bohl, Carsten Burk, Christoph Dümpelmann, Gerhard Feldhaus, Harald Gross, Didier Herman, Tania Hubenova, Max Keller, Helmut Jeske, Goran Klobucar, Pavel Kozák, Julia Langer, Andreja Lucic, Frank Meis, Ines Podszuck, Ronald Polivka, Miklós Puky, Przemyslaw Smietana, Steffen Zahn, and the Senckenberg Natural History Museum in Frankfurt, Germany. We are very grateful to Therese Bürgi, Jennifer Dahlem, and Kathrin Metzner for help in the laboratory. The study was financially supported by the Bundesministeriums für Ernährung, Landwirtschaft und Verbraucherschutz (BMELV) through the Bundesanstalt für Landwirtschaft und Ernährung (BLE).

## REFERENCES

Abrahamsson S.A.A., 1971. Density, growth and reproduction in populations of *Astacus astacus* and *Pacifastacus leniusculus* in an isolated pond. Oikos, 22, 373–380.

Agerberg A., 1990. Genetic variation in three species of freshwater crayfish; *Astacus astacus* L., *Astacus leptodactylus* Aesch., and *Pacifastacus leniusculus* (Dana), revealed by isozyme electrophoresis. Hereditas, 113, 101–108.

Alaranta A., Henttonen P., Jussila J., Kokko H., Prestegaard T., Edsman L. and Halmekyto M., 2006. Genetic differences among noble crayfish (*Astacus astacus*) stocks in Finland, Sweden and Estonia based on the ITS1 region. Bull. Fr. Pêche Piscic., 380-381, 965–975.

Albrecht H., 1983. Besiedlungsgeschichte und ursprüngliche holozäne Verbreitung der europäischen Flußkrebse (Decapoda: Astacidae). Spixiana, 6, 61–77.

Alderman D.J., 1996. Geographical spread of bacterial and fungal diseases of crustaceans. Rev. Sci. Tech. Off. Int. Epizoot., 15, 603–632.

Alderman D.J., Holdich D. and Reeve I., 1990. Signal crayfish as vectors in crayfish plague in Britain. Aquaculture, 86, 3–6.

Bandelt H.-J., Forster P. and Röhl A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol., 16, 37–48.

Bertocchi S., Brusconi S., Gherardi F., Grandjean F. and Souty-Grosset C., 2008. Genetic variability of the threatened crayfish *Austropotamobius italicus* in Tuscany (Italy): implications for its management. Fund. App. Lim., 173, 153–164.

Bott R., 1950. Die Flusskrebse Europas (Decapoda, Astacidae). Abh. Senckenberg. Naturf. Ges., 483, 1–36.

Bott R., 1972. Besiedlungsgeschichte und Systematik der Astaciden West-Europas unter besonderer Berücksichtigung der Schweiz. Rev. Suisse Zool., 79, 387–408.

Buhay J.E., 2009. "COI-like" sequences are becoming problematic in molecular systematic and DNA barcoding studies. J. Crustacean Biol., 29, 96–110.

Cataudella R., Paolucci M., Delaunay C., Ropiquet A., Hassanin A., Balsamo M. and Grandjeand F., 2010. Genetic variability of *Austropotamobius italicus* in the Marches region: implications for conservation. Aquatic Conserv. Mar. Freshw. Ecosyst. 20, 261–268.

Clement M., Posada D. and Crandall K.A., 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol., 9, 1657–1660.

Convention on Biological Diversity. Concluded at Rio de Janeiro on 5 June 1992, http://www.cbd.int/, accessed December 2010.

Crozier R.H., 1997. Preserving the information content of species: genetic diversity, phylogeny and conservation worth. Annu. Rev. Ecol. Syst., 28, 243–268.

Diéguez-Uribeondo J., Royo F., Souty-Grosset C., Ropiquet A. and Grandjean F., 2008. Low genetic variability of the white-clawed crayfish in the Iberian Peninsula: its origin and management implications. Aquat. Conserv. Mar. Freshwat. Ecosyst., 18, 19–31.

Durand J.D., Persat H. and Bouvet Y., 1999. Phylogeography and postglacial dispersion of the chub (*Leuciscus cephalus*) in Europe. Mol. Ecol., 8, 989–997.

Edsman L., Farris J.S., Kallersjo M. and Prestegaard T., 2002. Genetic differentiation between noble crayfish, *Astacus astacus* (L.) populations detected by microsatellite length variation in the rDNA ITS1 region. Bull. Fr. Pêche Piscic., 367, 691–706.

Edsman L., Füreder L., Gherardi F. and Souty-Grosset C., 2010. *Astacus astacus*. In:IUCN 2010, IUCN Red List of Threatened Species, Version 2010.4, http://www.iucnredlist.org, accessed December 2010.

Excoffier L., Smouse P.E. and Quattro J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial-DNA restriction data. Genetics, 131, 479–491.

Excoffier L., Laval G. and Schneider S., 2005. Arlequin version 3.11: An integrated software package for population genetics data analysis. Evol. Bioinform. Online, 1, 47–50.

Fevolden S.E., Taugbøl T. and Skurdal J., 1994. Allozymic variation among populations of noble crayfish, *Astacus astacus* L., in southern Norway: implications for management. Aquacult. Fish. Manage., 25, 927–935.

Grandjean F., Gouin N., Souty-Grosset C. and Diéguez-Uribeondo J., 2001. Drastic bottlenecks in the endangered crayfish species, Austropotamobius pallipes in Spain with inference to its colonization history. Heredity, 88, 1–8.

Hänfling B., Dümpelmann C., Bogutskaya N.G., Brandl R. and Brandle M., 2009. Shallow phylogeographic structuring of *Vimba vimba* across Europe suggests two distinct refugia during the last glaciation. J. Fish Biology, 75, 2269–2286.

Karaman M.S., 1962. Ein Beitrag zur Systematik der Astacidae (Decapoda). Crustaceana, 3, 173–191.

Karaman M.S., 1963. Studie der Astacidae (Crustacea, Decapoda) II. Teil. Hydrobiologia, 22, 111–132.

Keller M., 1999. Ten Years of Trapping *Astacus astacus* for Restocking in Lake Bronnen, a Gravel Pit in Bavaria. Freshwater Crayfish, 12, 518–528.

Koutrakis E., Perdikaris C., Machino Y., Savvidis G.and Margaris N., 2007. Distribution, recent mortalities and conservation measures of crayfish in hellenic freshwaters. Bull. Fr. Pêche Piscic., 385, 25–44.

Largiadèr C.R., Herger F., Lörtscher M. and Scholl A., 2000. Assessment of natural and artificial propagation of the white-clawed crayfish (Austropotamobius pallipes species complex) in the Alpine region with nuclear and mitochondrial markers. Mol. Ecol., 9, 25–37.

Maguire I., 2009. Die Flusskrebse Osteuropas. In:Füreder L. (ed.), Flusskrebse, Biologie – Ökologie – Gefährdung, Folio Verlag, Wien – Bozen, 92–97.

Moritz C., 1994. Defining 'evolutionary significant units' for conservation. Trends Ecol. Evol., 9, 373–375.

Nei M., 1987. Molecular Evolutionary Genetics, Columbia University Press, New York.

Östergren J., 2006. Migration and genetic structure of *Salmo salar* and *Salmo trutta* in northern Swedish rivers, Doctoral dissertation, Dept. of Aquaculture, SLU, Acta Universitatis agriculturae Sueciae.

Pullin A.S., Knight T.M., Stone D.A. and Charman K., 2004. Do conservation managers use scientific evidence to support their decision-making? Biol. Conserv., 119, 245–252.

Raymond M. and Rousset F., 1995. An exact test for population differentiation. Evolution 49, 1280–1283.

Ryder O.A., 1986. Species conservation and systematics: the dilemma of subspecies. Trends Ecol. Evol., 1, 9–10.

Sambrook J. and Russell D.W., 2001. Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, New York.

Schikora F., 1906. Die Krebspest. Fischerei-Zeitung, 9,529–532; 549–553; 561–566; 581–583.

Schulz H.K. and Grandjean F., 2005. Roundtable session 3: Phylogeny of European crayfish – improving the taxonomy of European crayfish for a better conservation. Bull. Fr. Pêche Piscic., 376-377, 829–836.

Schulz H.K., Smietana P. and Schulz R., 2004. Assessment of DNA variations of the noble crayfish (*Astacus astacus* L.) in Germany and Poland using inter-simple sequence repeats (ISSRs). Bull. Fr. Pêche Piscic., 372-373, 387–399.

Schulz R., 2000. Status of the noble crayfish *Astacus astacus* (L.) in Germany: monitoring protocol and the use of RAPD markers to assess the genetic structure of populations. Bull. Fr. Pêche Piscic., 356, 123-138.

Skurdal J. and Taugbøl T., 2002. *Astacus*. In: Holdich D.M.(ed.), Biology of Freshwater Crayfish, Blackwell Science Ltd, Oxford, 467-510.

Smietana P., Schulz H.K., Keszka S. and Schulz R., 2006. A proposal for accepting *Pontastacus* as a genus of European crayfish within the family Astacidae based on a revision of the west and east European taxonomic literature. Bull. Fr. Pêche Piscic., 380-381, 1041-1052.

Souty-Grosset C. and Reynolds J.D., 2009. Current ideas on methodological approaches in European crayfish conservation and restocking procedures. Knowl. Managt. Aquatic Ecosyst., 394-395, 01.

Souty-Grosset C., Grandjean F. and Gouin N., 2003. Involvement of genetics in knowledge, stock management and conservation of *Austropotamobius pallipes* in Europe. Bull. Fr. Pêche. Piscic., 370-371, 165-179.

Strayer D.L. and Dudgeon D., 2010. Freshwater biodiversity conservation: recent progress and future challenges. J. N. Am. Benthol. Soc., 29, 344–358.

Taberlet P., Fumagalli L., Wust-Saucy A.-G. and Cosson J.-F., 1998. Comparative phylogeography and postglacial colonization routes in Europe. Mol. Ecol., 7, 453-464.

Taubmann J., Theissinger K., Feldheim K., Laube I., Graf W., Johannesen J., Haase P. and Pauls S.U., 2011. Modelling range shifts and assessing genetic diversity distribution of the montane aquatic mayfly *Ameletus inopinatus* in Europe under climate change scenarios. Conserv. Genet., 12, 503-515.

Trontelj P., Machino Y. and Sket B., 2005. Phylogenetic and phylogeographic relationships in the crayfish genus *Austropotamobius* inferred from mitochondrial COI gene sequences. Mol. Phylogenet. Evol. 34, 212–226.

Weir B.S and Cockerham C.C., 1984. Estimating F-statistics for the analysis of population structure. Evolution, 38, 1358-1370.

Weiss S., Persat H., Eppe R., Schlötterer C. and Uiblein F., 2002. Complex patterns of colonization and refugia revealed for European grayling *Thymallus thymallus*,based on complete sequencing of the mitochondrial DNA control region. Mol. Ecol., 11, 1393-1407.

Zaikov A. and Hubenova T., 2007. Status of freshwater crayfish in Bulgaria. Proceedings III International conference "Fishery", 1–3 February 2007, Belgrade, 242–247.

Unpublished, first draft of the manuscript

# *Noble crayfish (Astacus astacus) with migratory background – natural versus human-mediated processes*

Short title: European recolonisation of noble crayfish

**A. Schrimpf[1], J. Dahlem[1], I. Maguire[2], L. Pârvulescu[3], H.K. Schulz[1], K. Theissinger[1], R. Schulz[1]**

[1] Institute for Environmental Sciences, University Koblenz-Landau, Fortstrasse 7, 76829 Landau, Germany
[2] Faculty of Scienece, University of Zagreb, Zagreb, Croatia
[3] Faculty of Chemistry, Biology, Geography, West University of Timisoara, Timisoara, Romania

## ABSTRACT

Conservation strategies aim to maintain the genetic diversity and the genetic integrity of a species. The phylogeographic history of a species can aid in defining areas of conservation priority. For freshwater species, the historical river structure plays a significant role in explaining the genetic differentiation and the population structure. Here, we aimed to reconstruct the post-glacial recolonization of central Europe of the endangered noble crayfish (*Astacus astacus*) and intended to identify refugial areas that are hotspots of genetic diversity. To address these questions, we analysed a fragment of the mitochondrial cytochrome oxidase subunit I (COI), and the 16S rRNA from 540 noble crayfish specimens from 158 sampling sites distributed throughout five European sea basins. Additionally, we conducted a microsatellite analysis of 289 individuals from 22 out of the 158 above-mentioned sites.

The haplotype diversity was highest in the Black Sea basin ($H_D$ = 0.851, 28 haplotypes) and lowest in the Baltic Sea basin ($H_D$ = 0.276, four haplotypes). The microsatellite data supported these results. Both markers revealed a particular high differentiation between populations from the Western Balkans to the remaining Black Sea populations. Western Balkan haplotypes diverged already around 710,890 years before present, whereas remaining differentiations occurred within the last 450,000 years before present. With migration modeling we detected that the North Sea basin and the Baltic Sea basin were colonized independently via different colonization paths from the eastern Black Sea basin,

while the Western Balkans did not contribute to the colonization. The results suggest the existence of at least two refugial areas in southeastern Europe. To conserve the maximum genetic diversity, the focus of conservation prioritization for noble crayfish should be set in southeastern Europe. We further propose that each river catchment forms a separate management unit.

**Key words**: mtDNA sequences • microsatellite analysis • refugial area • postglacial colonization • migration model • conservation management

## INTRODUCTION

The Convention on Biological Diversity (CBD) highlighted the preservation of biological diversity as one of its three main goals (SCBD 1992). According to the CBD, biological diversity includes the diversity of ecosystems, between species and within species (genetic diversity) (CBD Article 2). A high genetic diversity increases the chance of long-term survival because species with greater diversity are more likely to be able to survive in response to a changing environment compared to those with lower diversity (Malcolm *et al.,* 2007). Apart from the maintenance of the genetic diversity, the preservation of the native species' integrity (e.g. local specificity) is one of the major goals in conservation genetics (Souty-Grosset *et al*., 2003). To protect the integrity and the within-species diversity a detailed knowledge of the phylogenetic structure of a species is indispensable. Present-day genetic structure is a result of climatic variation and associated geomorphological and hydrographic conditions in the past.

### *Geological events in Europe*

Severe climatic oscillations and accompanying geomorphological and hydrographic conditions during the Pleistocene period resulted in great changes in species distribution areas characterised by contractions and expansions of their geographical ranges (Hewitt 1996). Southern Europe, especially the Iberian Peninsula, Apennine and the Balkans, served as a refuges during cold periods for many species (Hewitt 1999). During subsequent warm periods, a repeated northward expansion took place from refuges involving the leading edge (Hewitt 1996). Such a colonisation process of a limited number of individuals implies

successive founder effects that may lead to a loss of genetic diversity in the northern populations of cold-intolerant taxa (Taberlet *et al.,* 1998). The colonisation is followed by repeated extinctions of northern populations during cold climate phases (Hewitt 1996). In contrast, the diverse topography of southern refuges allows populations to diverge through several ice ages (Hewitt 1999).

The present distribution of freshwater organisms that often cannot migrate between river catchments has been most strongly influenced by the changes in landscapes caused by glaciers and melting water (e.g. Hänfling *et al.,* 2009; Vonlanthen *et al.,* 2007). The retreat of the glaciers formed the migration routes for freshwater species after the Last Glacial Maximum (LGM) that lasted from approx. 24,000 – 12,000 years before present (YBP). However, ephemeral contacts between river systems and changes in the flow direction of upper river systems complicate the reconstruction of the migration routes.

Over the LGM, freshwater crayfish were absent in central and northern Europe. They outlasted the glacial cycles on the Iberian Peninsula, the Italian Peninsula, the Balkan Peninsula or in the Ponto-Caspian region (Albrecht 1983). For noble crayfish (*Astacus astacus*), who probably survived the last glacial on the Balkan Peninsula (Albrecht 1983) under temperate conditions, a north- and westward spread along the Danube drainage system is most probable (Schulz and Grandjean, 2005) as it was shown for the freshwater fish species *Vimba vimba* (L, 1758) (Hänfling *et al.,* 2009), *Leuciscus cephalus* (L, 1758) (Durand *et al.,* 1999) or the European grayling *Thymallus thymallus* (L, 1758) (Weiss *et al.,* 2002). The recolonisation route reconstruction facilitates the identification of management units or evolutionary significant units (ESU) and is therefore relevant for conservation.

*Noble crayfish - threats*

The IUCN Red List of Threatened Species lists the noble crayfish as vulnerable with a decreasing population status (IUCN 2010). Besides environmental changes like water pollution or channelisation of streams, native crayfish species are highly threatened by invasive species. In particular, North American crayfish species pose a high threat to noble crayfish because they

potentially carry the oomycete fungus *Aphanomyces astaci* (Schikora 1906) that causes high mortalities in native crayfish populations (e.g. Alderman 1996). The high extinction rate goes along with a severe decrease in genetic diversity of European crayfish species. As the largest freshwater invertebrates and due to their trophic activities as omnivores, European crayfish play a key role in many freshwater ecosystems (Nyström 1999). The exctincion of local populations ay have drastic impacts on local biodiversity.

For more than 2000 years noble crayfish have been an object of commerce and trade (Skurdal and Taugbøl 2002) and are therefore influenced to the highest degree by human translocations (Albrecht 1983). The economic value of crayfish has led to noble crayfish stocking into numerous new localities. When the crayfish plague hit central Europe at the turn of the 19th century, noble crayfish were imported in large quantities from eastern European countries (Skurdal and Taugbøl 2002). Also today there are operating noble crayfish farms in several European countries, especially in central and northern Europe (e.g. Jussila and Mannonen 2004; Paaver and Hurt 2009) that sell crayfish as a food source, as stock for private ponds, or as donors for restocking programs in response to rapidly declining stocks. Stocking often occurs without knowing the taxonomic status of the stocking material (Souty-Grosset *et al.,* 2003). As a consequence, the trade leads to translocations and disintegrates the natural genetic structure. In addition, the construction of channels connecting different major rivers catchments also strongly influenced the distribution of crayfish species (Albrecht 1983).

*Previous study*

The first molecular-based phylogeographic study of noble crayfish by Schrimpf *et al.* (2011) covered a large part of the distribution range and resolved the large-scale genetic structure of this species: the haplotype diversity of the cytochrome oxidase subunit I (COI) fragment in central Europe was very low compared to the high diversity in the Balkan area where a centre of range expansion was suggested. Interestingly, a strong divergence between populations from the Western Balkans (Croatia) and the eastern Black Sea basin (Romania,

Bulgaria) was found (Schrimpf *et al.,* 2011). The genetic divergence may indicate the existence of two separate glacial refuges inhabited by populations that did not exchange genes for several centuries (Hewitt 1999). However, since only five specimens were analysed from the Western Balkans a final conclusion could not be drawn. The obtained result could also be the consequence of a human-mediated noble crayfish translocation. This first phylogeographic study of noble crayfish (Schrimpf *et al.,* 2011) has also shown that one marker (COI) is not sufficient to resolve the genetic structure of noble crayfish and additional markers are needed.

*Aim of this study*

In this study we analysed a second mtDNA marker (16S rRNA) to increase the resolution of the phylogenetic analyses. Although mtDNA is useful for studying historical lineage splits in the context of geological events, only little or no signal can be detected in bottlenecked populations that have recently colonised an area (Hewitt 1999). Therefore, we utilised nuclear microsatellite data to track the recent gene flow among populations and to estimate within population diversity. Moreover, we enhanced the number of sampling sites in southeastern Europe, especially in the Western Balkans. With this study we aim to answer three main questions that are fundamental for the conservation of the maximum genetic diversity of noble crayfish: a) Did the noble crayfish survive the last glacial in more than one refugial area in southeastern Europe? b) From which refugium did the noble crayfish recolonise central Europe? c) Was the Baltic Sea basin recolonised from the North Sea basin or from the Black Sea basin?

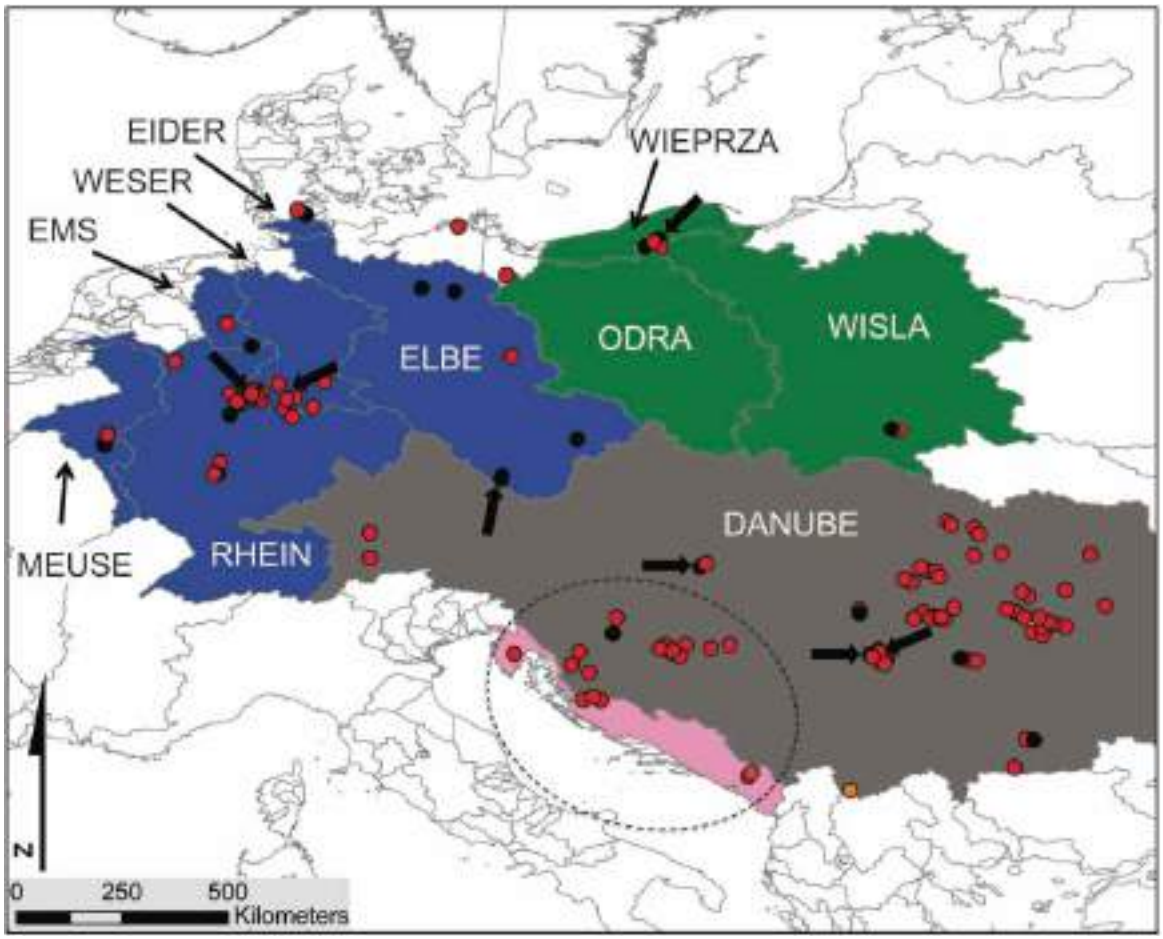


**Figure 1** Study area with sampling sites. Sea basins of European rivers are colored in blue for the North Sea basin, in green for the Baltic Sea basin, in grey for the Black Sea basin and in pink for the Adriatic Sea basin. The orange circle shows the one sample from the Aegean Sea that was included in the sequence analysis. Red circles indicate populations that were sequenced and black circles indicate populations that were additionally genotyped. When the black circle is overlaid by another circle, a bold arrow point to the hidden circle. Thin arrows point to the respective river catchments. The dotted circle indicates the populations grouped as 'Western Balkans'. The two sampling sites from Finland (Valkeinen and Ylä river catchments) are not shown.

## MATERIAL AND METHOD

*Sampling*

In total, 540 crayfish specimens from 158 sampling localities that are located within 14 river catchments (Figure 1, Table I in Supplementarly material) and from four hatcheries were collected by hand or by traps. The sampling sites were distributed within five different sea basins: 12 in the Baltic Sea basin, 59 in the North Sea basin, 87 in the Black Sea basin, four in the Adriatic Sea basin and one in the Aegean Sea basin. Immediately after the lower part of one pereopod

(propodus and dactylus) was taken specimens were released at the place where they were caught. Appendages usually regenerate after a few moults so the vitality of crayfish was not affected. Samples were stored in 70-96% Ethanol until DNA extraction. DNA was extracted from the muscle tissue using a standardized protocol ('Rapid isolation of Mammalian DNA', Sambrook and Russel, 2001).

*Laboratory work*

A 350 base pair (bp) fragment of cytochrome c oxidase subunit I (COI) and a 476 bp fragment of the 16S rRNA (16S), both mitochondrial DNA, were amplified using the primer pair ASTCOI (forward primer: GCGGGGATAGTAGGAACCTC; reverse primer: ATTTACCGCCCCTAAAATCG) and 16S_1471 and 16S_1472 (Crandall and Fitzpatrick 1996) respectively. The Polymerase chain reactions (PCR), the purification of the PCR products, the sequencing reaction and the sequencing were performed as described in Schrimpf *et al*. (2011). The annealing temperature in the PCR reaction for 16S was changed to 51° C. Sequences were edited and aligned with the SEQUENCHER 3.0 software (Gene Codes Corporation, Ann Arbor, Michigan, USA). Sequences were checked manually for base pair ambiguities, nuclear copies of mitochondrial derived genes, stop codons, and high levels of divergence, as recommended by Buhay (2009). All haplotypes were submitted to GenBank (Accessions numbers will be provided upon acceptation of the manuscript). As both COI and 16S share the same history, sequences were concatenated and treated as a single locus for the following analyses except for the calculation of the Bayesian tree.

For 289 individuals from 22 populations from 12 river catchments and the hatchery in Augsburg (Table 1 in supplementary material) a total of six microsatellite loci with a dinucleotide repeat were analysed using the species-specific primer pairs Aas2, Aas6, Aas8, Aas11, Aas766, Aas1198 (Koiv *et al.,* 2008, 2009). Forward primers were designed with a 19 bp M13-tail that was labelled during the PCR reaction using a universal fluorochrome-labelled M13 primer. PCR were carried out using a Primus 96 Cycler (Peqlab Biotechnologie GmbH, Erlangen, Germany) under the following conditions: An initial denaturation at 95°C for 2 min, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at

72°C, and a final extension of 5 min at 72°C. The 60°C annealing was replaced by 63°C for primer Aas11 and by 57°C for primer Aas8. 2 µl PCR-product were added to 30 µl SLS (Beckman Coulter, Krefeld, Germany). The fragment analysis was performed on a Beckman Coulter CEQ 8000 eight capillary sequencer. Loci were scored using the software CEQ SYSTEM version 9 (Beckman Coulter, Krefeld, Germany). 10% of all samples were randomly chosen and repeated for estimation of the error rate (Bonin *et al.,* 2004).

*Statistical analysis - Sequence analyses*

The statistical analyses were calculated either for sea basins (e.g. North Sea basin) or river catchments (e.g. Rhine catchment) or for both. From the Black Sea basin we have only samples from the Danube river catchment, therefore the same samples were used for a sea basin comparison (as 'Black Sea basin') and for a river catchment comparison ('as Danube river catchment'). Genetic variation within sea basins and river catchments were measured in terms of the number of haplotypes ($H_N$), haplotype diversity ($H_D$) and number of segregating sites using DNASP v 5.10.1 (Librado and Rozas 2009). To detect genetic differentiation a hierarchical analysis of molecular variance (AMOVA, Excoffier *et al.,* 1992) with populations from each river catchment partitioned into separate groups was performed and $\Phi_{ST}$-values among sea basins and river catchments were estimated with ARLEQUIN v 3.11 (Excoffier and Lischer 2010). Significance was based on 1000 random permutations. To identify haplotypes and to determine the phylogenetic relationships between haplotypes a median joining (MJ) network (Bandelt *et al.,* 1999) was constructed using the software NETWORK 4.610 (Fluxus Technology, Suffolk, UK). DNASP v 5.10.1 was used to perform a mismatch analysis, which plots the distribution of the number of differences between pairs of sequences and compares it with a fit to an unimodal Poisson distribution as expected under sudden expansion from a small population. An unimodal pattern in the graph indicates that a species underwent a recent population expansion. A multimodal, ragged pattern in the graph, however, indicates that this species has maintained a stable population size over a long period of time. The value of the calculated raggedness statistic r (Harpending *et al.,* 1993) is lower in expanding population compared with a constant population

size. To test for deviation of sequence variation from evolutionary neutrality Tajima's *D* and Fu's F tests (Tajima 1989; Fu 1997) were carried out with ARLEQUIN v 3.11. Under a scenario of sudden expansion these values will be negative, and positive in a population that's suffered a recent decrease in population size (Excoffier *et al.,* 1992).

A phylogenetic tree was constructed using BEAST version 1.7.3 (Drummond *et al.,* 2012). The molecular clock test was performed in MEGA v 5.05 (Tamura *et al.,* 2011). The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level ($P < 1.12$). Therefore, we used a relaxed uncorrelated lognormal clock model. Lacking fossil data to calibrate a species specific molecular clock, we used a rate divergence for COI of 1.4% per million years considered as an approximate average divergence rate reported for crustaceans (Knowlton and Weight, 1998) that was recently used for the white-clawed crayfish (Pedraza-Lara *et al.,* 2010). Since no divergence rate is available for 16S we used an estimated molecular clock for 16S. We used a HKY substitution model (Hasegawa *et al*., 1985) with gamma distributed rate heterogeneity and Optimum heterogeneity parameters with empirical base frequencies, and 5 rate categories for both partitions as proposed by Treefiner (Jobb 2011). The relative fit of each model to the data was assessed using the Akaike Information Criteria (AIC, Akaike 1973). The starting tree was randomly generated with a Birth-and-Death process prior on the tree. The Markov Chain Monte Carlo (MCMC), 400 million steps long, was performed sampling every $10{,}000^{th}$ generation. The BEAST-run was visualized and diagnosed with Tracer version 1.5 (MCMC Trace Analysis Tool, Rambaut and Drummond, 2009). TreeAnnotator version 1.7.3 (Drummond and Rambaut 2011) was used for summarising the information in a sample of trees onto a single 'target' tree whereas the first 10% of the samples were discarded as burn-in. The consensus tree was finally put into graphs with FigTree version 1.3.1 (Rambaut 2009). The cold climate phases as revealed by benthic oxygen isotope stages ($\delta^{18}O$, Lisiecki and Raymo 2005) were incorporated into the tree figure.

To compare the probability of different dispersal routes, we applied a model selection approach implemented in MIGRATE-N v 3.3.2 (Beerli and Felsenstein

2001). To keep computation feasible the, 152 sampling sites were pooled according to geographic patterns (sea basins) and previous results. We tested ten hypotheses that were translated into migration matrices. The first genealogy was started using a random tree. Initial theta and migrant values were generated from a $F_{ST}$ calculation. A static heating scheme with four different temperatures was applied. We ran 1 mio generations, from which 50,000 were sampled after a burn-in period of 10,000. For the log-equivalent Bayes factor (LBF) estimation we used the thermodynamic integration (T.I.) approximation using a Bezier-curve because it results in LBFs with high repeatability and little variance (Beerli and Palczewski 2010). A high T.I. value indicates a better fit of the model than a low T.I..

*Statistic analysis - Microsatellite analyses*

For the nuclear DNA markers the GENETIC DATA ANALYSIS (GDA) v 1.1 software (Lewis and Zaykin 2001) was used to calculate the number of private alleles ($A_P$) per sea basin, river catchment and population. The number of alleles ($A_N$) and the expected ($H_E$) and observed ($H_O$) heterozygosity for the sea basins, the river catchments and populations were performed in ARLEQUIN version 3.5.1.3 (Excoffier and Lischer 2010). The ARLEQUIN software was further used to perform an AMOVA (Excoffier *et al.,* 1992) with populations from each river catchment partitioned into separate groups, and to calculate $F_{ST}$-values between river catchments and sea basins. The structure of and the variation among all genotyped individuals were determined by a factorial correspondence analysis (FCA) with the default settings in GENETIX 4.05 (Belkir *et al.,* 1996-2004). The genetic population partitioning was evaluated using the Bayesian clustering approach in the program BAPS v 5.4 (Corander *et al.,* 2008). We used predefined sampling units whereas all individuals of a population form one group. The program was initially run with a fixed number of clusters (K = 22). The analysis was repeated five times to determine to most likely number of K. With a probability of 1 the number of K was 15. In a second analysis, five runs, each at K = 10 to K = 20, were performed to confirm the number of clusters.

## RESULTS

*Sequence data*

The 540 sequences of the COI and 16S genes resulted in 30 and 15 different haplotypes, respectively. COI was more diverse with $H_D = 0.548$ and 27 polymorphic sites, and 16S was less diverse with $H_D = 0.410$ and 15 polymorphic sites. The concatenated sequences with a length of 826 bp resulted in 46 different haplotypes with 42 polymorphic sites. The mean $H_D$ for the entire sample set was 0.626. The diversity values for the sea basins suggested that populations of the Black Sea basin were highly diverse ($H_D = 0.851$) representing 28 haplotypes (Table 1). The Adriatic Sea basin is also characterised by a high genetic diversity ($H_D = 0.576$, five haplotypes), whereas it has to be noted that only 12 samples from the Adriatic Sea basin were included. The North Sea basin had a lower haplotype diversity ($H_D = 0.316$) and less haplotypes (13). The Baltic Sea basin had the lowest genetic diversity ($H_D = 0.276$, four haplotypes).

The results of the hierarchical AMOVA are shown in Table 2. Test indicated that the majority of variance was present among populations within river catchments (62.50%, $p < 0.001$). In total, 25.33% ($p < 0.001$) is attributed to variation within populations while variation was moderate between river catchments (12.16%, $p < 0.05$). The $\Phi_{ST}$-values for mtDNA suggested that the sea basins were significantly different (p-values < 0.05; Table 3). The $\Phi_{ST}$-values between the Aegean Sea basin (N = 1) on the one side and the North and Baltic Sea basins on the other side are high ($\Phi_{ST} = 0.789$ and 0.943) but not significant. With regard to the river catchments, noble crayfish from the Eider were significantly different from all river catchments except the Valkeinen river catchment (Table 4).

**Table 1** Results from the combined COI and 16S mitochondrial sequences for the analysed sea basins (BAS = Baltic Sea, NS = North Sea, BS = Black Sea, AdS = Adriatic Sea; above line) and the river catchments (below line). The Black Sea basin is also treated as a river catchment (Danube). Given are the number of sequenced individuals (N), the number of haplotypes ($H_N$), the haplotype diversity ($H_D$) (calculated with DNASP v 1.10.1) and the number of polymorphic sites (S), values for Tajima´s D, Fu´s F and Raggedness (r) (calculated with ARLEQUIN v 3.11). Significant values are indicated in bold ($p < 0.05$). The samples from the Aegean Sea basin (AeS) are excluded due to the low sample size (N = 1). Presented are the proposed groups for the calculations with MIGRATE-N (F = Finland, EBS = eastern Black Sea basin, WB = Western Balkans).

| Sea basin | River catchment | Migrate | Sites | N | $H_N$ | $H_D$ | S | Tajima´s D | Fu´s F | r |
|---|---|---|---|---|---|---|---|---|---|---|
| BAS | | | 14 | 66 | 5 | 0.276 | 4 | -1.39 | **-3.34** | 0.30 |
| NS | | NS | 59 | 249 | 13 | 0.316 | 24 | **-1.93** | **-4.88** | 0.36 |
| BS | | EBS/ WB | 82 | 185 | 28 | 0.851 | 32 | -0.79 | **-6.29** | 0.03 |
| AdS | | WB | 5 | 12 | 5 | 0.576 | 14 | -0.52 | 1.58 | 0.23 |
| BAS | Odra | BAS | 1 | 5 | 1 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| BAS | Uecker | BAS | 1 | 3 | 1 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| BAS | Wieprza | BAS | 2 | 10 | 1 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| BAS | Wisla | BAS | 3 | 15 | 3 | 0.362 | 2 | -1.00 | -0.92 | 0.19 |
| BAS | Valkeinen | F | 1 | 5 | 2 | 0.600 | 1 | 1.22 | 0.626 | 0.4 |
| BAS | Ylä | F | 1 | 5 | 1 | 0 | 0 | 0.00 | 0.00 | 0.00 |
| NS | Eider | NS | 1 | 9 | 2 | 0.222 | 1 | -1.09 | -0.26 | 0.36 |
| NS | Elbe | NS | 6 | 39 | 3 | 0.194 | 13 | **-1.53** | 3.36 | 0.68 |
| NS | Ems | NS | 1 | 5 | 2 | 0.400 | 3 | -1.05 | 1.69 | 0.68 |
| NS | Meuse | NS | 2 | 9 | 2 | 0.389 | 1 | 0.16 | 0.48 | 0.20 |
| NS | Rhine | NS | 41 | 176 | 11 | 0.349 | 18 | **-1.82** | -2.17 | 0.48 |
| NS | Weser | NS | 3 | 11 | 1 | 0 | 0 | 0.00 | 0.00 | 0.00 |

**Table 2** Results of the analysis of molecular variance (AMOVA) calculated with ARLEQUIN v 3.11 based on mtDNA and microsatellite allele frequencies where populations are grouped according to European river catchments. The percentage of the total variance (% Var), fixation indices and their significance (*: $p < 0.05$ ***: $p < 0.001$) based on 1000 random permutations are shown. pop. = populations, Micsat = Microsatellite data.

| DNA marker | Among groups | | Among pop. within groups | | Within pop. | |
|---|---|---|---|---|---|---|
| | % | F-statistic | % | F-statistic | % | F-statistic |
| mtDNA | 12.16 | 0.122* | 62.5 | 0.712*** | 25.33 | 0.747*** |
| Micsat | 15.89 | 0.159*** | 29.85 | 0.354*** | 54.26 | 0.457*** |

**Table 3** Pairwise FST- and ΦST-values between European sea basins as implemented in ARLEQUIN v 3.11. Results for microsatellites are shown above the diagonal, mtDNA data is shown below the diagonal (for abbreviation see Table 1). Significant values are indicated in bold ($p < 0.05$).

| | **BAS** | **NS** | **BS** | **AdS** | **AeS** |
|---|---|---|---|---|---|
| BAS | 0.000 | **0.365** | **0.283** | - | - |
| NS | **0.071** | 0.000 | **0.231** | - | - |
| BS | **0.170** | **0.162** | 0.000 | - | - |
| AdS | **0.855** | **0.789** | **0.438** | 0.000 | - |
| AeS | 0.943 | 0.789 | 0.162 | 0.291 | 0.000 |

**Table 4**Pairwise $F_{ST}$- and $\Phi_{ST}$-values between river catchments and Finland calculated with Arlequin v 3.11. Results for microsatellite data is shown above the diagonal and mtDNA data is shown below the diagonal. Significant values are indicated in bold ($p < 0.05$).

| | **Ems** | **Elbe** | **Eider** | **Meu.*** | **Wes.*** | **Rhine** | **Uec.*** | **Wie.*** | **Odra** | **Wis.*** | **Dan.*** | **Val.*** | **Ylä** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Ems** | 0.000 | **0.311** | **0.181** | **0.460** | - | 0.008 | - | **0.171** | **0.293** | **0.199** | **0.179** | **0.165** | **0.195** |
| **Elbe** | -0.019 | 0.000 | **0.207** | **0.095** | - | **0.217** | - | **0.273** | **0.224** | **0.462** | **0.341** | **0.157** | **0.215** |
| **Eider** | **0.656** | **0.422** | 0.000 | **0.429** | - | **0.168** | - | **0.149** | **0.096** | **0.422** | **0.302** | 0.038 | **0.122** |
| **Meu** | 0.092 | 0.008 | **0.771** | 0.000 | - | **0.304** | - | **0.556** | **0.382** | **0.723** | **0.400** | **0.286** | **0.377** |
| **Wes.*** | 0.170 | -0.014 | **0.910** | 0.156 | 0.000 | - | - | - | - | - | - | **-** | - |
| **Rhine** | -0.086 | **0.057** | **0.507** | 0.053 | 0.018 | 0.000 | - | **0.163** | **0.249** | **0.188** | **0.201** | **0.127** | **0.169** |
| **Uec.*** | -0.132 | -0.166 | **0.845** | -0.068 | 0.000 | -0.119 | 0.000 | - | - | - | - | **-** | - |
| **Wie.*** | 0.149 | -0.020 | **0.905** | 0.141 | 0.000 | 0.013 | 0.000 | 0.000 | **0.230** | **0.378** | **0.281** | 0.091 | **0.134** |
| **Odra** | 0.000 | -0.080 | **0.870** | 0.034 | 0.000 | -0.040 | 0.000 | 0.000 | 0.000 | **0.502** | **0.347** | **0.102** | **0.230** |
| **Wis.*** | 0.169 | 0.022 | **0.804** | 0.112 | -0.013 | 0.051 | -0.173 | -0.019 | -0.084 | 0.000 | **0.346** | **0.260** | **0.280** |
| **Dan.*** | 0.031 | **0.105** | **0.263** | **0.116** | **0.116** | **0.162** | 0.003 | **0.112** | 0.067 | **0.131** | 0.000 | **0.255** | **0.254** |
| **Val.*** | -0.071 | 0.032 | **0.763** | 0.231 | 0.441 | 0.48 | 0.118 | 0.417 | 0.250 | 0.225 | 0.667 | 0.000 | 0.035 |
| **Ylä** | 0.000 | -0.080 | **0.870** | 0.034 | 0.000 | -0.040 | 0.000 | 0.000 | 0.000 | -0.062 | **0.110** | 0.250 | 0.000 |

River catchments abbreviationed as following: Meu. - Meuse, Wes. - Weser, Uec. - Uecker, Wie. - Wieprza, Wis. - Wisla, Dan. - Danube, Val. - Valkeinen

The network showed a star-like pattern around the most common haplotype (Hap01, Figure 2) that was found in 327 samples distributed throughout the sampling area except in the Adriatic and Aegean Sea basins. Additionally, the network revealed a strong branching around two haplotypes that are common in the Black Sea basin only. A group of haplotypes from the Western Balkans (Hap41 – Hap46) formed a subsection with a distance of at least 8 bp to all other haplotypes. Four haplotypes were shared between North Sea and Baltic Sea basins, two between Adriatic Sea and Black Sea basins, one between the Baltic Sea (Finland) and the Black Sea basin and one between North Sea, Black Sea

and Baltic Sea basins. We found private haplotypes in all sea basins, seven in the North Sea basin three in the Baltic Sea basin, three in the Adriatic Sea basin, one in the Aegean Sea basin (N = 1) and 24 in the Black Sea basin, of which four were found only in the hatchery in Augsburg (Table I in Supplementarly material).

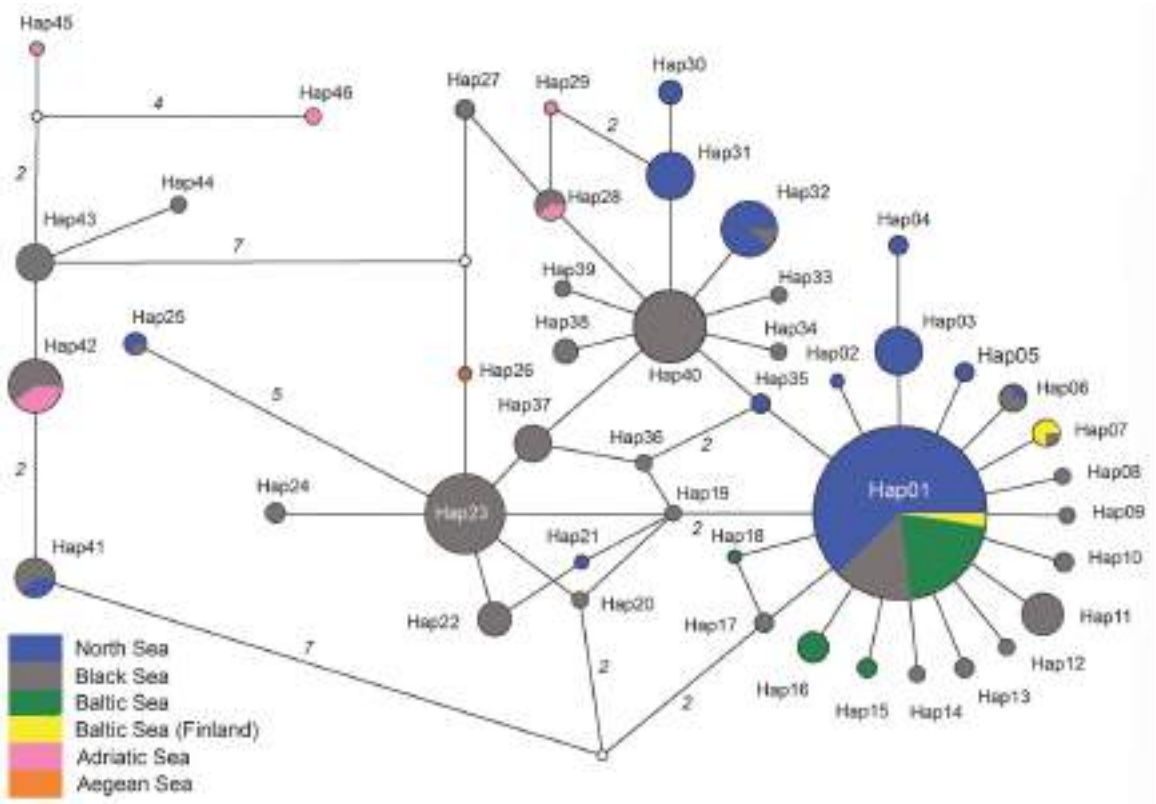


**Figure 2** Median joining network constructed using NETWORK 4.610 for 826 bp of the concatenated COI- and 16S-sequences reveals genealogical relationships among 540 *Astacus astacus* samples. Each connecting branch line represents nucleotide substitutions. The number of mutational steps is given above the branch, except when it equals 1. Hollow circle represent missing haplotypes inferred from mutational changes, but absent in this data set. The size of circles is proportional to the frequency of the represented haplotype. Haplotype codes on the network correspond to samples listed in Table 1. Finnish samples correspond to the Baltic Sea basin, but were highlighted because of their high geographic differentiation to the other Baltic Sea basin samples.

The unimodal ragged pattern in the pair-wise differences graph of the mismatch analysis for the whole data set was shifted to the left of the distribution (graph not shown) and very closely matched the expected distribution for a recent population expansion (Rogers and Harpending 1992). Raggedness statistic for the whole data set was low and not significant ($r = 0.088$, $p = 0.428$) and therefore we cannot reject the hypothesis of population expansion. Mismatch distribution for

the sea basin groups revealed unimodal patterns for the North Sea and Baltic Sea basins, while multimodal and ragged distributions were detected for the Black Sea and Adriatic Sea basin (graphs are not shown). Fu´s F was significant negative ($p < 0.05$) for the Baltic Sea and Black Sea basin and Fu´s F and Tajima´s D were both significant ($p < 0.05$) for the North Sea basin (Table 1). With regard to the river catchments, Tajima´s D was significant negative for populations from the Rhine and Elbe river catchments (Tajima´s D = -1.82 and -1.53, respectively; $p < 0.05$).

In the haplotype phylogeny inferred from the combined COI and 16S sequences haplotypes clustered into two well-supported major clades (posterior probability, pp = 1) (Figure 3). The lineage 4 contained six haplotypes from the Adriatic Sea and Black Sea basin (all Western Balkans). Haplotypes from the Black Sea basin were present in all four lineages and haplotypes from the North Sea basin in all lineages except in the lineage 4. Haplotypes from the Baltic Sea basin cluster only within one clade (lineage 1). Most splits were not significant. However, the split between lineage 4 and lineages 2 – 3 and between lineage 3 and 1 – 2 is highly supported (pp = 1 and 0.93, respectively).

For the model selection calculated with MIGRATE-N, we tested several combinations of sample groups. Due to the genetic similarity and the relatively small geographic distance between populations from the south-western Black Sea basin (Croatia) and the Adriatic Sea basin, we pooled this data (dotted circle in Figure 1). This group will be referred to as 'Western Balkans' and the remaining Black Sea basin as 'eastern Black Sea basin'. The Aegean Sea basin was excluded from this analysis due to the low sampling size. Because of the high geographic distance, we further separated Finland from the Baltic Sea basin. The remaining geographic group was North Sea basin. The probability values/ Bayes factors preferred the model 2b (T.I. = -2071.61) (Figure 4). This model implies a bifurcal colonization route from the eastern Black Sea basin to a) the North Sea basin and to b) the Baltic Sea basin. According to the favoured model the eastern Black Sea basin was colonised from the Western Balkans and the Finnish sampling sites were colonized from the Baltic Sea basin.

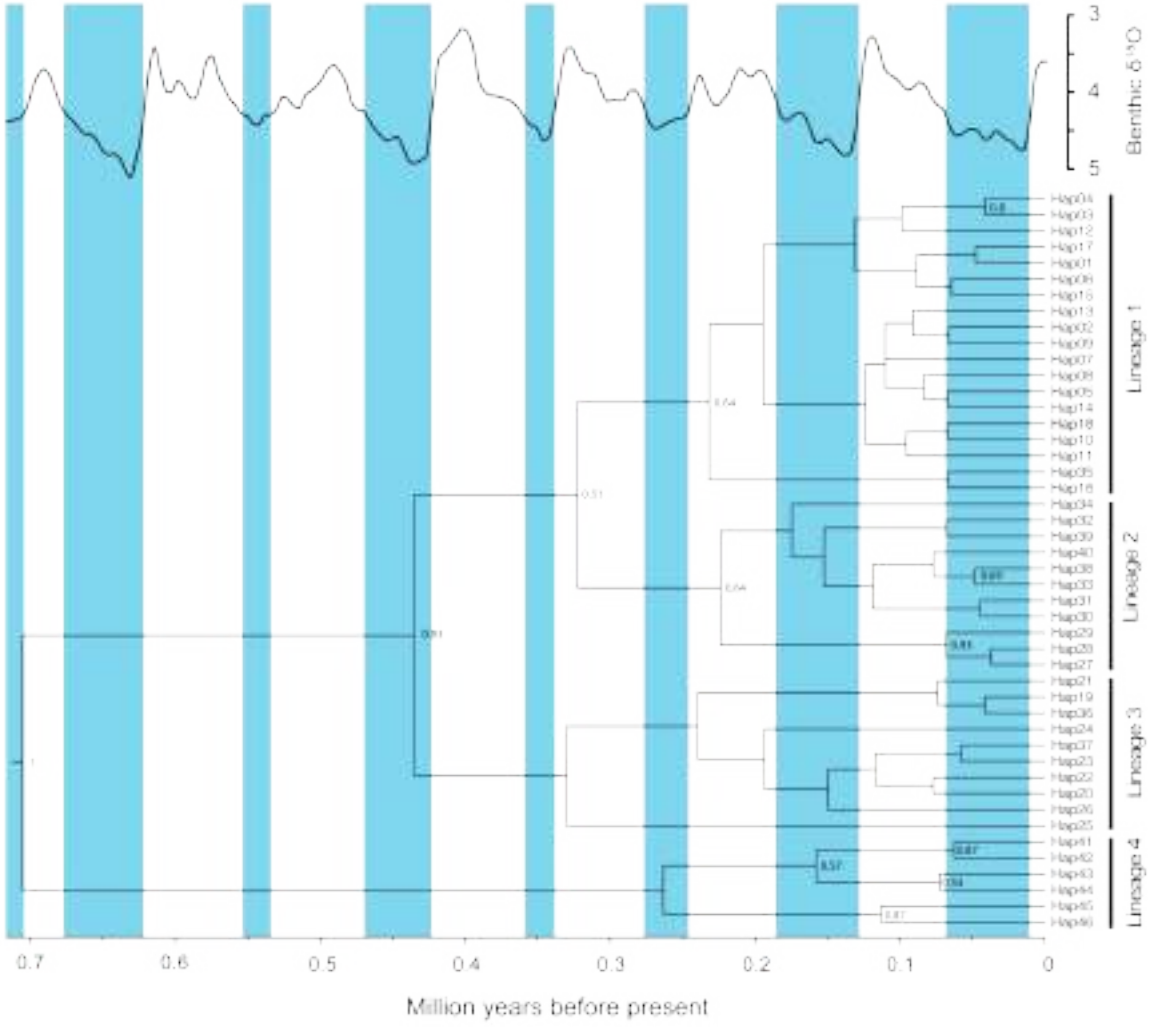


**Figure 3** Benthic oxygen isotope stages ($\delta^{18}O$; Lisiecki and Raymo, 2005; top oft the figure) and haplotype phylogeny generated using Bayesian Markov Chain Monte Carlo analysis as implemented in BEAST. The calculation was based on a combined 350 bp fragment of the COI-sequence and a 476 bp fragment of the 16S sequence. The axis shows the time scale in million years before present. Number at the nodes are the posterior probability values above 0.5.

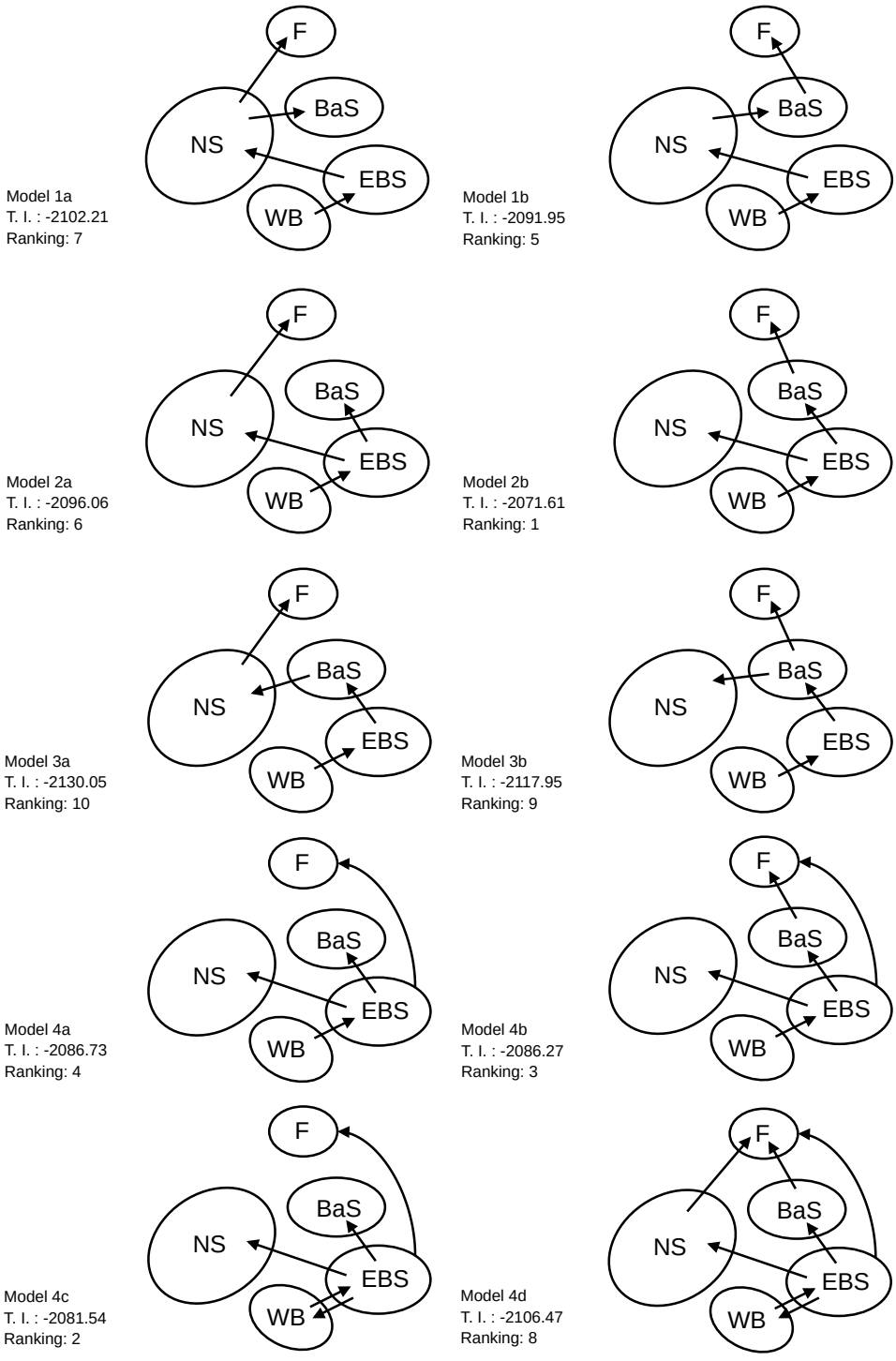


**Figure 4** Results of the migration hypothesis model selection determined with MIGRATE-N v 3.3.2 between different regions according to geographic regions and genetic results. Finland (F) was separated from the Baltic Sea basin (BaS) and Croatia was separated from the Black Sea basin and pooled with samples from the Adriatic Sea basin to form the group 'WB' = Western Balkan. The remaining Black Sea basin samples form the 'EBS' = eastern Black Sea. NS = North Sea basin. Arrows indicate the directions of gene-flow. Thermodynamic Integration (T.I.) gives the fit of the model. The ranking from 1 to 10 for the models is stated. Note that only ten samples were included from Finland.

*Microsatellite data*

A genotyping error rate of 1.8% was estimated and should thus not bias our results. A total of 100 alleles with an average of 16.67 alleles per locus were observed. The highest $H_E$ (0.799) and the highest number of alleles per number of samples analysed ($A_N/N$ = 0.92) were detected in the Black Sea basin (Table 5). The North Sea basin ($H_E$ = 0.543) is indicated by a higher heterozygosity value than the Baltic Sea basin ($H_E$ = 0.414), but by a lower number of alleles compared to the number of analysed samples ($A_N/N$ = 0.49) (Table 5). With regard to the individual populations, the populations from the Black Sea have higher expected heterozygosity values (mean $H_E$ = 0.456) than the populations from the North Sea basin (mean $H_E$ = 0.320) and Baltic Sea basin (mean $H_E$ = 0.231).

In the hierarchical AMOVA tests with populations from each river catchment partitioned into separate groups the majority of variation was present within populations (54.26%, $p < 0.001$; Table 2), variation was moderate within river catchments (29.85%, $p < 0.001$) and minor among river catchments (15.89%, $p < 0.001$). The $F_{ST}$-values suggested that the sea basins were significantly differentiated ($p < 0.05$; Table 3). All river catchments among and within sea basins differed significantly from each other apart from the Rhine and the Ems ($F_{ST}$ = 0.008, $p < 0.05$; Table 4), the Valkeinen and Eider ($F_{ST}$ = 0.030, $p < 0.05$) and the Valkeinen and Wieprza.

The first two factorial components of the FCA explain 10.98% of the variance of the microsatellite data. The data points from the Black Sea basin individuals are distributed most widespread in the FCA graph (Figure 5) indicating a high genetic variation. The data points from the North Sea basin are more widespread than those from the Baltic Sea basin. There is an overlap of individuals from the North Sea and Baltic Sea basin. Data points from the Western Balkan samples clearly group apart from all other samples.

The Bayesian cluster analysis based on river catchments selected a clustering of K = 15 with a probability of 0.92 as the best model explaining the population structure (Table 5). The geographically distant Finnish populations (Baltic Sea basin) form one cluster. The hatchery in Augsburg groups in the same cluster as one population from the Rhine and one from the Ems River catchment.

One population from the Elbe, the Meuse and the Rhine form another cluster. Furthermore, two Elbe populations group in one cluster and one Wieprza population and one Eider population in another. For the remaining 11 populations (all from the Danube River catchment) each population was considered as a unique group.

**Table 5** Results from the microsatellite analysis for the analysed sea basins (above line, for abbreviation see Table 1) and sampling sites (below line). The number of genotyped individuals (N), the number of private alleles ($A_P$) and private alleles per N ($A_P$/N), the number of alleles ($A_N$) and alleles per N ($A_N$/N) and the expected ($H_E$) and observed heterozygosity ($H_O$) calculated with ARLEQUIN v 3.11 are given. The same capital letter (A-F) in the column 'Cluster' indicates populations that belong to the same cluster as evaluated using the Bayesian clustering approach in the program BAPS v 5.4. The 'x' indicates populations that form their own cluster.

| | Sampling site | River catchment | Sea basin | N | $A_N$ | $A_N$/N | $A_p$ | $A_p$/N | $H_E$ | $H_O$ | Cluster |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sea basin | | | BAS | 62 | 39 | 0.63 | 4 | 0.11 | 0.414 | 0.156 | |
| | | | NS | 124 | 61 | 0.49 | 10 | 0.08 | 0.543 | 0.228 | |
| | | | BS | 79 | 73 | 0.92 | 35 | 0.44 | 0.799 | 0.319 | |
| River catchment | Rakowe Duze | Odra | BAS | 15 | 16 | 1.07 | 3 | 0.20 | 0.239 | 0.160 | x |
| | Bez Nazwu | Wieprza | BAS | 9 | 13 | 1.45 | 1 | 0.11 | 0.247 | 0.239 | A |
| | Czarne | Wisla | BAS | 11 | 11 | 1.00 | 0 | 0.00 | 0.153 | 0.100 | x |
| | Valkeinen | Kymen | BAS | 13 | 13 | 1.00 | 0 | 0.00 | 0.226 | 0.161 | E |
| | Ylä | Kokemäenjoki | BAS | 14 | 13 | 1.00 | 0 | 0.00 | 0.291 | 0.218 | E |
| | Florenville | Meuse | NS | 15 | 10 | 0.67 | 0 | 0.00 | 0.197 | 0.046 | B |
| | Jäglitz | Elbe | NS | 7 | 9 | 1.29 | 0 | 0.00 | 0.147 | 0.074 | B |
| | Svetlohor | Elbe | NS | 10 | 12 | 1.20 | 0 | 0.00 | 0.216 | 0.147 | C |
| | Kramata | Elbe | NS | 8 | 11 | 1.37 | 1 | 0.13 | 0.218 | 0.128 | C |
| | U sudu | Elbe | NS | 13 | 17 | 1.31 | 0 | 0.00 | 0.377 | 0.244 | D |
| | Ambach | Rhein | NS | 18 | 25 | 1.39 | 0 | 0.00 | 0.491 | 0.456 | D |
| | Merzkrebse | Rhein | NS | 5 | 12 | 2.40 | 0 | 0.00 | 0.296 | 0.200 | B |
| | Dauphe | Rhein | NS | 19 | 35 | 1.84 | 6 | 0.32 | 0.531 | 0.368 | F |
| | Gut Riedberg | Ems | NS | 15 | 23 | 1.53 | 0 | 0.00 | 0.503 | 0.344 | F |
| | Langsee | Eider | NS | 14 | 15 | 1.07 | 1 | 0.07 | 0.220 | 0.165 | A |
| | Clocotici | Danube | BS | 8 | 22 | 2.75 | 7 | 0.88 | 0.511 | 0.494 | x |
| | Gorna Trape | Danube | BS | 22 | 20 | 0.91 | 1 | 0.05 | 0.387 | 0.349 | x |
| | Vukovina Lake | Danube | BS | 6 | 16 | 2.67 | 7 | 1.17 | 0.550 | 0.306 | x |
| | Ciornovãt | Danube | BS | 5 | 16 | 3.20 | 6 | 1.20 | 0.479 | 0.283 | x |
| | Kádárta | Danube | BS | 4 | 10 | 2.51 | 0 | 0.00 | 0.327 | 0.125 | x |
| | Dragoiestilor | Danube | BS | 22 | 27 | 1.23 | 5 | 0.23 | 0.452 | 0.355 | x |
| | Nadas | Danube | BS | 12 | 18 | 1.50 | 1 | 0.08 | 0.487 | 0.353 | x |
| | Augsburg | Hatchery | | 24 | 32 | 1.33 | 1 | 0.04 | 0.550 | 0.439 | F |

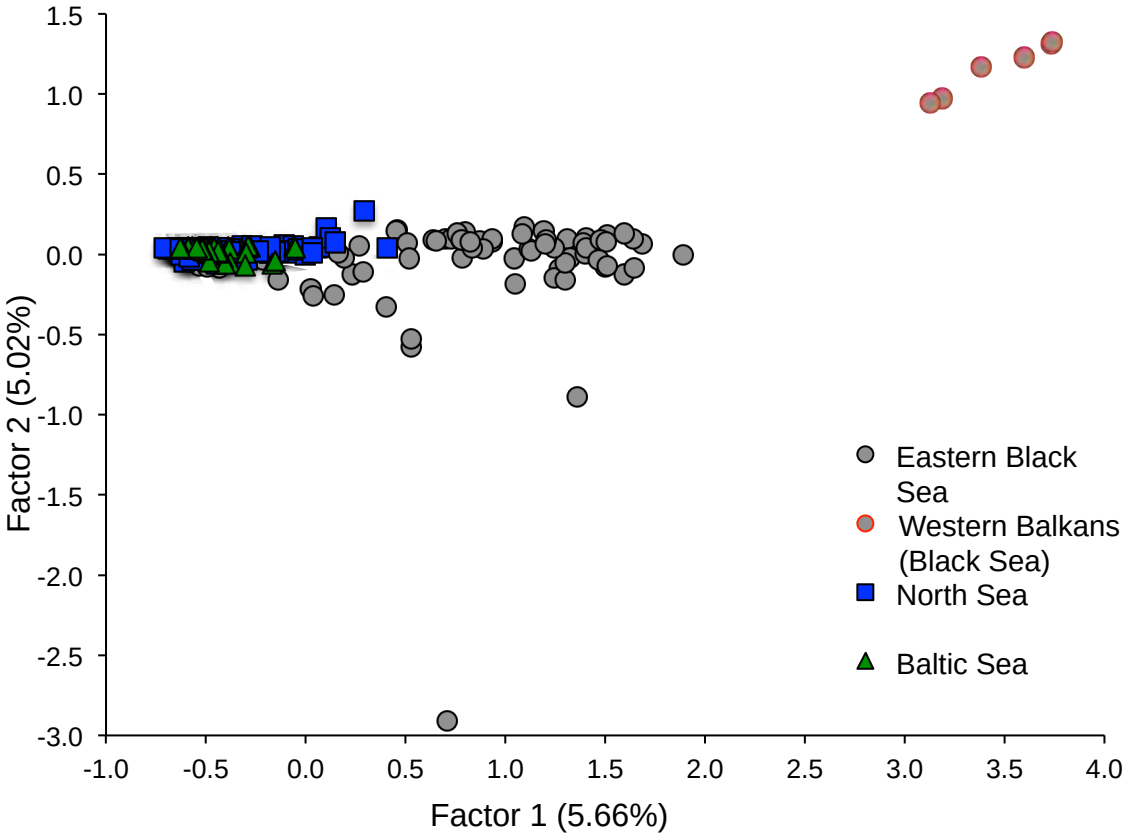


**Figure 5** Factorial Correspondence Analysis over determined using GENETIX showing the degree of similarity of the 289 *Astacus astacus* individuals across six microsatellite loci based on the first two dimensions (factors). Each dot represents one individual. The distance between points reflects the degree of genetic differentiation among individuals.

## DISCUSSION

Conservation strategies aim to maintain the genetic diversity and the genetic integrity of a species. The phylogeographic history of a species can aid in defining areas of conservation priority. Here, we conducted a phylogeographic analysis of noble crayfish to identify their genetic hotspots and to reconstruct the colonisation history of this native European species. Our results support the hypothesis that noble crayfish outlasted the last glacial cycles in southeastern Europe in at least two refugia. The mtDNA analysis suggests a bifurcated colonization process from the eastern Black Sea basin to a) the North Sea and b) the Baltic Sea basin.

*Glacial survival in southeastern Europe*

The high diversity of mtDNA and nuclear DNA indicated that southeastern Europe is the hotspot of genetic diversity of noble crayfish. In contrast, very low genetic diversities of both markers were detected in central Europe (Table 1 and 5). It is known that high levels of population differentiation characterise older populations that have already diverged before the last ice age (Hewitt 1999). Therefore, we suggest that the high genetic differentiation in southeastern Europe evolved during several glacial cycles when this area served as a glacial refuge while climate conditions were unfavourable in central and northern Europe. In particular, we observed a high genetic differentiation between the Western Balkans and the eastern Black Sea basin (Romania and Bulgaria), suggesting that noble crayfish survived in at least two glacial refuges in southeastern Europe. This long separation between both refuges is supported by the results of the Bayesian phylogenetic tree where haplotypes from the Western Balkans form a distinct lineage (Lineage 4, Figure 3) that split already around 711,000 YBP from eastern Black Sea basin haplotypes. Because of the higer differentiation of haplotyopes from the Western Balkans, it might be possible that the Western Balkans was an older glacial refuge for noble crayfish. Eventualy, the Western Balkans was less affected than the eastern Balkans by adverse climate conditions. The Western Balkans is the region with the highest genetic diversity for many species (e.g. Bănărescu 2004; Sket *et al.,* 2004). High genetic diversity in the stone crayfish (*Austropotamobius torrentium*) was detected by COI sequence analysis in the Kolpa / Kupa drainage along the border between Slovenia and Croatia (Trontelj *et al.,* 2005). The high genetic diversity in the Western Balkans and in the eastern Black Sea basin as well as the high differentiation among these areas revealed by both markers supports the hypothesis of at least two refugia in southeastern Europe during the LGM.

An additional Southwest-European refugium might explain the high differentiation of haplotypes from Rhineland-Palatinate (North Sea basin; Hap30 – Hap32) to the remaining North Sea samples. It is conceivable that besides the Danube River basin there existed other refuges in Europe during the LGM. Noble crayfish were presumably present in central Europe before the last ice age, and

when the climate cooled again they could have retreated besides to a southeastern European refugium also to a Western refugium. The LGM they could have outlasted in some periodically ice-free rivers in southwestern Europe as assumed for the fish species *Cottus gobio* (L, 1758) that might have survived the LGM in today´s eastern France (Vonlanthen *et al.,* 2007). However, this theory can not be supported and the differentiated haplotypes might also be a result of artificial translocation.

The divergence times in Figure 3 show that the separation of the major lineages is not related to the cold climate phases. While genetic divergence of other species took place when subpopulations were geographically isolated in different refuges (Hetitt 1999), this correlation could not be detected for noble crayfish. However, the $\delta^{18}O$ curve contains data from globally distributed sites (Lisiecki and Raymo 2005) and does not consider regional variation. Further, the divergence rate of 1.4% per million years for COI has to be considered with caution because it is not specific for freshwater crayfish species. The lack of hierarchical structure in the phylogenetic tree can be explained by a low number of base exchanges in the mtDNA caused by a founder effect of noble crayfish in Central and Northern Europe. The low genetic variation might also explain the star-like pattern around three common haplotypes in the haplotype network.

*Recolonisation of central Europe*

In contrast to southeastern Europe, a homogeneous gene pool dominates in the North and Baltic Sea basin (Table 1). The low genetic diversities are characteristic for a more recently colonised area (Taberlet *et al*., 1998). In this study we have revealed a significant negative value for both Tajima´s D and Fu´s F for the North Sea basin (Table 1) that indicate a recent population expansion. Extinctions of noble crayfish in northern and central Europe followed by founder effects probably led to the homogenization of noble crayfish in northern and central Europe. The endemic haplotypes from the Western Balkan populations are highly differentiated from the remaining haplotypes (Figure 2 and 3). This genetic separation is also supported by the FCA analysis of the nuclear data where the Western Balkans data points grouped separately from all other

samples (Figure 5). Possibly the Alps hindered the direct spread of the Western Balkan lineages into central Europe. Additionally, as supposed for other species (Hewitt 2000) the early colonisation from another refugia prevented the expansion of noble crayfish from the Western Balkans. We rather show that the eastern Black Sea basin 'pool' (e.g. Hap 01) contributed to the post-glacial recolonisation of noble crayfish from the eastern Black Sea basin into central and northern Europe as it was demonstrated for many other freshwater species (e.g. Hänfling *et al.,* 2009; Durand *et al.,* 1999; Weiss *et al.,* 2002). In the phylogenetic tree and in the MJ network haplotypes from the eastern Black Sea group together with haplotypes from the North and Baltic Sea basins. This was also confirmed by the model selection calculated with MIGRATE-N. For the calculation the Western Balkans was separated as a distinct group. The favoured model points to the bifurcated colonization route from the eastern Black Sea basin to a) the North Sea basin and to b) the Baltic Sea basin (Figure 4). For the colonisation of Finland, the model with a colonisation route from the Baltic Sea basin further to Finland was favoured. However, the colonisation from the Black Sea basin to Finland (second highest probability value) is also possible, presumably via the Don and Volga Rivers. Nevertheless, the sampling size of Finnish samples was too small (N = 10) to finally reveal this question. The $F_{ST}$-values (Table 3) confirm the result of the model selection. The higher differentiation between North Sea and Baltic Sea indicates a longer separation than between Black Sea and the North Sea, respectively Baltic Sea basin. However, it has to be mentioned, that from southeastern Europe besides from the Western Balkans we have only data from Romania and Bulgaria. It is very well possible that the glacial refuge was even further south, e.g. in Greece or Macedonia, and Romania and Bulgaria were recolonised after the LGM from a more southern refuge before noble crayfish recolonised central and northern Europe. This is also supported by the heterogenous distribution of haplotypes in Romania indicating that the haplotypes did not evolve in the side channels of the Danube in Romania. An artificial translocation explaining this genetic structure can be excluded (Albrecht 1983).

From a potential western refugium noble crayfish could have recolonised Rhineland-Palatinate. A survival of noble crayfish in northern German rivers

during the LGM could further explain the private haplotypes in the Eider River catchment. For example European grayling is believed to have persisted the LGM in the northern Elbe and/or Vistula River catchment (Gum *et al.,* 2005). Therefore, it might be possible that also noble crayfish were not totally absent in central Europe during the last glacial period. This scenario would contradict earlier assumptions (e.g. Albrecht 1983; Schulz and Grandjean 2005). However, these samples could also be a result of stocking events. The origin of samples from France should be subject of further investigations.

*Incongruent distribution of haplotypes*

Along with the influences of climatic variation, historical river structure plays a significant role in explaining the genetic differentiation and the population structure of many freshwater species (Abellán *et al.,* 2012). The finding of the same haplotypes in the Danube and Elbe (Hap25) or in the Danube and Rhine River catchment (Hap32) could be explained by channels that were modified by periglacial melt water and have changed water flow direction between and within drainages caused by geological events (Hantke 1993). Otherwise the geographical incongruent distribution could be explained by an artificial translocation across river catchments by men. Human influence can be assumed, for example, for the distribution of the very distinct haplotype Hap41 with 10 base pairs differing from the most frequent haplotype Hap01. Hap41 was found in Croatia and in the geographically very distant Elbe River catchment. The results from the microsatellite data also indicate a disturbed distribution of noble crayfish. The cluster analysis did not assign the populations from the same river catchments into one cluster as it is expected for species with a natural genetic structure. The hatchery in Augsburg formed one cluster with a population from the Rhine (Dauphte) and the Ems River catchments (Gut Riedberg). This might be a result of noble crayfish stocking from the hatchery in Augsburg to these waters. It is well known that translocations of noble crayfish for commercial purposes have been conducted for centuries (Skurdal and Taugbøl 2002) but were intensified in the last century in response to rapidly declining crayfish stocks (Souty-Grosset *et al*., 2003). Former translocations have been detected with genetic methods for other crayfish species, e.g. *Austropotamobius pallipes* (Grandjean *et al.,* 2002) or

*Austropotamobiu italicus italicus* (Pedraza-Lara *et al*., 2010). For noble crayfish, the artificial translocation might have increased the local diversity, but destroyed the natural genetic structure of this species, especially in central Europe where the artificial influence on the noble crayfish distribution was higher than in southeastern Europe (Albrecht 1983). The translocation further decreases the overall genetic diversity of noble crayfish because local and endemic haplotypes and alleles may be lost.

According to the results from the genetic differentiation analyses ($\Phi_{ST}$-values, $F_{ST}$-values), nuclear DNA and mtDNA reveal contradicting results. Contrary to the results based on mtDNA, with nuclear DNA a relatively high differentiation between the Black Sea and the North Sea as well as between the Black Sea and the Baltic Sea was observed (Table 3). The incongruence is common in phylogenetic studies because the two markers have a different mode of inheritance (Zink and Barrowclough 2008). Possible explanations are: a) The high evolutionary rate in nuclear DNA can lead to a high differentiation between some populations. At the same time it can lead to homoplasy in different alleles and result in an underestimation of genetic diversity and genetic differentiation. In this case ancestral polymorphism might not be detected anymore (Theissinger *et al.,* 2011). b) Translocation can cause a discrepancy between the two markers, especially when a translocated population is mixed with a native population.

As can be seen by the high number of missing haplotypes in the MJ network, many haplotypes are either extinct or were not included in the sampling. In particular, many haplotypes that link the eastern and Western Balkans haplotypes are missing. A more detailed analysis of southeastern European populations should be conducted and the number of populations for a microsatellite analysis should be increased in order to reveal the local hotspots of diversity and to define eastern European ESUs.

*Implications for conservation*

Compared to other European freshwater crayfish species where a higher number of base pair exchanges was revealed in the COI sequence (e.g. *Astacus leptodactylus*; Maguire pers. com.; *Austropotamobius torrentium* and *A. pallipes*,

Trontelj *et al*., 2005), noble crayfish exhibit a small genetic diversity. A good conservation management of noble crayfish is still lacking and strongly needed in whole Europe to minimize the loss of genetic diversity. This is especially of crucial importance in the Danube River catchment that is subject of a recent invasion process of American crayfish species and the crayfish plague (Hudina *et al.,* 2009; Pârvulescu *et al.*, 2012; Schrimpf *et al.,* 2012). Between-catchment translocations for economic reasons and conservation programs that consist of restocking crayfish without knowledge of their taxonomic status (Souty-Grosset *et al.,* 2003) both have negative effects on the conservation of the genetic diversity and the genetic integrity of noble crayfish. It is claimed that in restocking projects for crayfish species the donor population should be selected based on its local genetic distinctiveness. If no money for genetic analysis is available, the results of this study (inter-basin diversity) support the idea of conservation of catchment-specific gene pools (Weiss *et al.,* 2002) and each river catchment should be managed as a distinct ESU. In a genetic analysis an indication for an unnatural origin is given by incongruent distributed haplotypes (e.g. Hap41 in the Elbe River catchment) or significant distinct alleles in a population. When there are several options for a local donor population the ones with a high genetic diversity should be selected (Taugbøl and Peay 2004). Even if the genetic structure of noble crayfish is already partly dissolved, following these suggestions the maximum diversity of the species and the integrity of local populations will be preserved.

## ACKNOWLEDGEMENT

We thank the following persons for providing samples: Erik Bohl, Carsten Burk, Christoph Dümpelmann, Gerhard Feldhaus, Harald Gross, Didier Herman, Tania Hubenova, Sylvia Idelberger, Max Keller, Helmut Jeske, Goran Klobucar, Pavel Kozák, Julia Langer, Andreja Lucic, Frank Meis, Ines Podszuck, Ronald Polivka, Miklós Puky, Sascha Schleich, Przemyslaw Smietana, Steffen Zahn. The study was partly financially supported by the Bundesministeriums für Ernährung, Landwirtschaft und Verbraucherschutz (BMELV) through the Bundesanstalt für Landwirtschaft und Ernährung (BLE) (Förderkennzeichen: 07BM019).

## REFERENCES

Akaike H (1973) Information Theory and an Extension of the Maximum Likelihood Principle. In: Petrov BN, Csaki F, editors. Second International Symposiumon Information Theory. Budapest: Akademia Kiado. pp. 267–281.

Albrecht H (1983) Besiedlungsgeschichte und ursprüngliche holozäne Verbreitung der europäischen Flußkrebse (Decapoda: Astacidae). Spixiana, 6: 61-77.

Alderman DJ (1996) Geographical spread of bacterial and fungal diseases of crustaceans. Rev Sci Tech Off Int Epizoot, 15: 603–632.

Abellán P, Arribas P and Svenning J-C (2012) Geologic habitat template overrides late Quaternary climate change as a determinant of range dynamics and phylogeography in some habitat-specialist water beetles. J Biogeogr, 39: 970-983.

Bănărescu PM (2004) Distribution pattern of the aquatic fauna of the Balkan Peninsula. In: Balkan biodiversity: Pattern and process in the European hotspot (Eds H.I. Griffiths, B. Kryštufek & J.M. Reed), pp. 203-219. Kluwer Academic Publishers, Dordrecht.

Beerli P. and M. Palczewski, 2010. Unified framework to evaluate panmixia and migration direction among multiple sampling locations. Genetics, 185: 313-326.

Beerli P and Felsenstein J (2001) Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. Proc Natl Acad Sci USA, 98: 4563–4568.

Belkhir K, Borsa P, Chikhi L, Raufaste N and Bonhomme F (1996-2004) GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5171, Université de Montpellier II, Montpellier (France).

Bonin A, Bellemain E, Bronken Eidesen P, Pompanon F, Brochmann C and Taberlet P (2004) How to track and assess genotyping errors in population genetic studies. Mol Ecol, 13: 3261–3273.

Brandelt H-J, Forster P and Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16: 37–48.

Buhay JE (2009) "COI-like" sequences are becoming problematic in molecular systematic and DNA barcoding studies. J Crustacean Biol. 29: 96–110.

Corander J, Sirén, J and Arjas E (2008) Bayesian spatial modeling of genetic population structure. Comput Stat, 23: 111–129.

Crandall KA and JF Fitzpatrick Jr (1996) Crayfish molecular systematics: Using a combination of procedures to estimate phylogeny. Syst Biol, 45.1: 1-26.

Drummond AJ, Suchard MA, Xie D and Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol, 29: 1969-1973.

Durand JD, Persat H and Bouvet Y (1999) Phylogeography and postglacial dispersion of the chub (*Leuciscus cephalus*) in Europe. Mol Ecol, 8: 989–997.

Excoffier L, Smouse PE and Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial-DNA restriction data. Genetics, 131: 479–491.

Excoffier L and HEL Lischer (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour. 10: 564-567.

Fu Y (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics, 147: 915–925.

Grandjean F, Frelon-Raimond M and Souty-Grosset C (2002) Compilation of molecular data for the phylogeny of the genus Austropotamobius*:* one species or several? Bull Fr Pêche Piscic, 367: 671-680.

Gum B, Gross R and Kuehn R (2005). Mitochondrial and nuclear DNA phylogeography of European grayling (*Thymallus thymallus*): evidence for secondary contact zones in central Europe. Mol Ecol, 14: 1707–1725.

Hantke R (1993) Flussgeschichte Mitteleuropas. Ferdinand Enke-Verlag, Stuttgart.

Hänfling B, Dümpelmann C, Bogutskaya NG, Brandl R and Brandle M (2009) Shallow phylogeographic structuring of *Vimba vimba* across Europe suggests two distinct refugia during the last glaciation. J Fish Biology,75: 2269–2286.

Harpending HC, Sherry ST, Rogers AR and Stoneking M (1993) The genetic structure of ancient human populations. Curr Anthropol, 34: 483–496.

Hasegawa M, Kishino H and Yano T (1985): Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol, 22: 160-174.

Hewitt GM (1996) Some genetic consequences of ice ages and their role in divergence and speciation. Biol J Linn Soc, 58: 247-276.

Hewitt GM (1999) Post-glacial re-colonization of European biota. Biol J Linn Soc, 68: 87–112.

Hewitt (2000) The genetic legacy of the Quaternary ice ages. Nature, 405: 907-913

Hudina S, Faller M, Lucić A, Klobučar G, Maguire I (2009) Distribution and dispersal of two invasive crayfish species in the Drava River basin, Croatia. Knowl Manag Aquat Ecosyst, 394−395: 9.

IUCN (2012) IUCN Red List of Threatened Species. Version 2012.2. Available at: www.iucnredlist.org (accessed 10 December 2012).

Jobb G (2011) TREEFINDER version of March 2011. Munich, Germany. (computer program). Retrieved from www.treefinder.de.

Jussila J and Mannonen A (2004) Crayfisheries in Finland, a short overview. Bull Fr Pêche Piscic, 372–373: 263–273.

Knowlton N and Weight LA (1998) New dates and new rates for divergence across the Isthmus of Panama. Proc R Soc Lond Ser B: Biol Sci, 265: 2257–2263.

Koiv K, Gross R, Paver T and Kuehn R (2008) Isolation and characterization of first microsatellite markers for the noble crayfish, *Astacus astacus*. Conservation Genetics 9: 1703-1706.

Koiv K, Gross R, Paver T and Kuehn R (2009) Isolation and characterization of 11 novel microsatellite DNA markers in the noble crayfish, *Astacus astacus*. Animal Genetics 40: 124.

Lewis PO and Zaykin D (2001) Genetic Data Analysis: Computer program for the analysis of allelic data. Version 1.0 (d16c). (computer program). Retrieved from www.lewis.eeb.uconn.edu/lewishome/software.html.

Librado P and Rozas J (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25: 1451-1452.

Lisiecki LE and ME Raymo (2005) A Pliocene-Pleistocene stack of 57 globally distributed benthic δ18O record. Paleoceanography, 20: PA1003.

Malcolm L, Hunter Jr and James P (2007) Fundamentals of Conservation Biology. 3rd edition. Blackwell Publishing, Oxford, United Kingdom, pp 516.

Nyström P (1999) The effects of crayfish on interactions in freshwater benthic communities. PhD thesis, Lund University, Lund, Sweden.

Paaver T and Hurt M (2009) Status and management of noble crayfish Astacus astacus in Estonia. Knowl Manag Aquat Ecosyst, 394-395: 18.

Pârvulescu L, Schrimpf A, Kozubíková E, Resino SC, Vrålstad T, Petrusek A, Schulz R (2012) Invasive crayfish and crayfish plague on the move: first detection of the plague agent *Aphanomyces astaci* in the Romanian Danube. Dis Aquat Org, 98: 85-94.

Pedraza-Lara C, Alda F, Carranza S and Doadrio I (2010) Mitochondrial DNA structure of the Iberian populations of the white-clawed crayfish, *Austropotamobius italicus italicus* (Faxon, 1914). Mol Phyl Evol, 57: 327-342.

Rambaut A and Drummond AJ (2011) TreeAnnotator version 1.7.3 (computer program). Retrieved from www.beast.bio.ed.ac.uk.

Rambaut A (2009) FigTree version 1.3.1 (computer program). Retrieved from www.beast.bio.ed.ac.uk.

Rambaut A and Drummond AJ (2009) Tracer version 1.5 (computer program). Retrieved from www.beast.bio.ed.ac.uk.

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. Mol Biol Evol, 9: 552–569.

SCBD (Secretariat of the Convention on Biological Diversity) (1992) Convention on Biological Diversity. Available at: www.cbd.int (accessed 29 December 2012).

Schrimpf A, Pârvulescu L, Copila-Ciocianu D, Petrusek A, Schulz R (2012) Crayfish plague pathogen detected in the Danube Delta – a potential threat to freshwater biodiversity in southeastern Europe. Aquat Invas, 7 (4): 503–510.

Schrimpf A, Schulz H, Theissinger K, Pârvulescu L, Schulz R. (2011) First large-scale genetic analysis of the vulnerable noble crayfish *Astacus astacus* reveals low haplotype diversity of central European populations. Knowl Manag Aquat Ecosyst, 401: 35.

Schulz HK and Grandjean F (2005) Phylogeny of European crayfish – improving the taxonomy of European crayfish for a better conservation. Bull Fr Pêche Piscic, 376-377: 829-83.

Sket B, Paragamian K and Trontelj P (2004) A census of the obligate subterranean fauna of the Balkan Peninsula. In: Balkan biodiversity: Pattern and process in the European hotspot (ed. HI Griffiths, B Kryštufek and JM Reed), pp. 309-322. Kluwer Academic Publishers, Dordrecht.

Skurdal J and Taugbøl T (2002) *Astacus*. In: Holdich DM (ed.), Biology of Freshwater Crayfish, Blackwell Science Ltd, Oxford, United Kingdom, 467–510.

Souty-Grosset C, Grandjean F and Gouin N (2003) Involvement of genetics in knowledge, stock management and conservation of *Austropotamobius* in Europe. Bull Fr Pêche Piscic, 370-371: 165-179.

Taberlet P, Fumagalli L, Wurst-Saucy A-G, Cosson J-F (1998) Comparative phylogeography and postglacial colonization routes in Europe. Mol Ecol, 7: 453-464.

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics, 123: 585–595.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol, 28: 2731−2739

Taugbøl T and Peay S (2004) Roundtable Session 3. Reintroduction of native crayfish and habitat restoration. Bull Fr Pêche Piscic, 372-373: 465–471.

Theissinger KT (2011) Comparative phylogeography of two co-distributed arctic-alpine freshwater insect species in Europe. PhD Thesis, Johannes Gutenberg University, Mainz

Trontelj P, Machino Y and Sket B (2005) Phylogenetic and phylogeographic relationships in the crayfish genus *Austropotamobius* inferred from mitochondrial COI gene sequences. Mol Phylogenet Evol, 34: 212–226.

Vonlanthen P, Excoffier I, Bittner D, Persat H, Neuenschwander S and Largiadèr CR (2007) Genetic analysis of potential postglacial watershed crossings in Central Europe by the bullhead (*Cottus gobio* L.). Mol Ecol, 16(21): 4572-4584

Weiss S, Persat H, Eppe R, Schlötterer C and Uiblein F (2002) Complex patterns of colonization and refugia revealed for European grayling *Thymallus thymallus*, based on complete sequencing of the mitochondrial DNA control region. Mol Ecol, 11: 1139–1407.

Zink RM and Barrowclough GF (2008) Mitochondrial DNA under siege in avian phylogeography. Molecular Ecology, 17: 2107–2121.

## SUPPLEMENTARY MATERIAL

Table I Description of the sampling locations and haplotypes of analyzed *Astacus astacus*. Given is the number of genotyped (Ms N) and sequenced (Seq N) individuals per sampling site, number of the most common haplotype (Hap01) and the number of all other haplotypes (Hap). The sampling sites are descibed by the respective sea basin (SB: BAS = Baltic Sea, NS = North Sea, BS = Black Sea; AdS = Adriatic Sea, AeS = Aegean Sea), the longitude and latitude (N°, E°), the river catchment (RC, KO = Kokemäenjoki, DA = Danube) and country (GER = Germany, PO = Poland, F =, France CZ = Czech Republic, BE = Belgium, BU = Bulgaria, A = Austria, HU = Hungary, RO = Romania, HR = Croatia, MNE = Montenegro, KOS = Kosovo). The geographical coordinates of the study sites can be obtained from the corresponding author.

| Population (site) | SB | Country | RC | Ms N | Seq N | Hap01 | Hap |
|---|---|---|---|---|---|---|---|
| Koppelsee | BAS | GER | Uecker | | 3 | 3 | |
| Barthe | BAS | GER | | | 5 | | Hap16(5) |
| Tonkuhle | BAS | GER | | | 3 | 3 | |
| Rakowe Duze | BAS | PO | Odra | 15 | 5 | 5 | |
| Seki | BAS | PO | Wieprza | | 5 | 5 | |
| Bez Nazwu | BAS | PO | Wieprza | 9 | 5 | 5 | |
| Czarne | BAS | PO | Wisla | 11 | 5 | 5 | |
| Rosko | BAS | PO | Wisla | | 5 | 2 | Hap15(2)<br>Hap18(1) |
| Tomczyna | BAS | PO | Wisla | | 5 | 5 | |
| Lake1 close to Miastko | BAS | PO | | | 5 | 5 | |
| Lake2 close to Miastko | BAS | PO | | | 5 | 5 | |
| Lake3 close to Miastko | BAS | PO | | | 5 | 5 | |
| Valkeinen | BAS | F | Kymen | 14 | 5 | 3 | Hap07(2) |
| Ylä | BAS | F | KO | 15 | 5 | 5 | |
| Gut Rietberg | NS | GER | Ems | 15 | 5 | 4 | Hap32(1) |
| Langsee | NS | GER | Eider | 14 | 9 | | Hap03(8)<br>Hap04(1) |
| Stepenitz | NS | GER | Elbe | | 5 | 5 | |
| Schwarze Elster | NS | GER | Elbe | | 6 | 6 | |
| Jäglitz | NS | GER | Elbe | 9 | 7 | 5 | Hap41(2) |
| Svetlohor | NS | CZ | Elbe | 11 | 8 | 8 | |
| U sudu | NS | CZ | Elbe | 15 | 6 | 4 | Hap25(2) |
| Kramata | NS | CZ | Elbe | 8 | 7 | 7 | |
| Florenville | NS | BE | Meuse | 15 | 5 | 5 | |
| Libramont | NS | BE | Meuse | | 4 | 2 | Hap13(2) |
| Aar | NS | GER | Rhine | | 5 | 5 | |
| Allna | NS | GER | Rhine | | 4 | 3 | Hap21(1) |
| Ambach | NS | GER | Rhine | 12 | 5 | 5 | |
| Mühlgraben Caldern | NS | GER | Rhine | | 3 | 2 | Hap06(1) |
| Dautphe | NS | GER | Rhine | 20 | 4 | 4 | |
| Dielbach (Woog) | NS | GER | Rhine | | 8 | 3 | Hap05(1)<br>Hap31(3)<br>Hap41(1) |
| Donsbach | NS | GER | Rhine | | 5 | 5 | |
| Eichelbach | NS | GER | Rhine | | 5 | 5 | |
| Fischbach | NS | GER | Rhine | | 6 | 3 | Hap30(3) |

| Population (site) | SB | Country | RC | Ms N | Seq N | Hap01 | Hap |
|---|---|---|---|---|---|---|---|
| Fohnbach | NS | GER | Rhine | | 5 | 5 | |
| Giebelsbach | NS | GER | Rhine | | 1 | 1 | |
| Gansbach | NS | GER | Rhine | | 5 | 5 | |
| Geierstein/Roth | NS | GER | Rhine | | 10 | 10 | |
| Waldteich, Irrschelde | NS | GER | Rhine | | 5 | 5 | |
| Kallenbach | NS | GER | Rhine | | 5 | **5** | |
| Lasterbach | NS | GER | Rhine | | 5 | 5 | |
| Merzkrebse | NS | GER | Rhine | 5 | 5 | 5 | |
| Meerbach | NS | GER | Rhine | | 1 | 1 | |
| Madenmühlen | NS | GER | Rhine | | 5 | 5 | |
| Mademühlen 2 | NS | GER | Rhine | | 4 | 4 | |
| Nanzenbach | NS | GER | Rhine | | 1 | 1 | |
| Klausbach | NS | GER | Rhine | | 7 | 3 | Hap32(4) |
| Mahlscheid | NS | GER | Rhine | | 6 | 6 | |
| Perf | NS | GER | Rhine | | 5 | 5 | |
| Hartmann/Reh-bach | NS | GER | Rhine | | 5 | 5 | |
| Pollichia Woog | NS | GER | Rhine | | 2 | | Hap32(2) |
| Pollichia Woog2 | NS | GER | Rhine | | 3 | 1 | Hap32(2) |
| Schlettenbachtal | NS | GER | Rhine | | 1 | | Hap32(1) |
| Wolfsägertal | NS | GER | Rhine | | 7 | 2 | Hap05(1)<br>Hap31(2)<br>Hap32(2) |
| Meisertalweiher | NS | GER | Rhine | | 5 | 5 | |
| Saarbach (Lagerweiher) | NS | GER | Rhine | | 1 | 1 | |
| Saarbacher Mühlweiher | NS | GER | Rhine | | 2 | | Hap31(2) |
| Saarbach/Saar-bachhammer | NS | GER | Rhine | | 2 | | Hap31(2) |
| Saarbach (Woog) | NS | GER | Rhine | | 5 | 4 | Hap31(1) |
| Salzbach | NS | GER | Rhine | | 5 | 5 | |
| Eifel, S. Schleich | NS | GER | Rhine | | 2 | 1 | Hap32(1) |
| Saarbacher Hammer | NS | GER | Rhine | | 1 | | Hap31(1) |
| Spielberg | NS | GER | Rhine | | 5 | 2 | Hap32(3) |
| Steinbruch Rot | NS | GER | Rhine | | 5 | 5 | |
| Stippbach | NS | GER | Rhine | | 5 | 5 | |
| Waldteich bei Wallenfels | NS | GER | Rhine | | 5 | 4 | Hap02(1) |
| Breitweiher/Rhön | NS | GER | Weser | | 2 | 2 | |
| Ocherbach | NS | GER | Weser | | 5 | 5 | |
| Urff | NS | GER | Weser | | 4 | 4 | |
| Razdvec | BS | BU | DA | | 6 | 4 | Hap12(1)<br>Hap14(1) |
| Beli Osam | BS | BU | DA | | 4 | | Hap11(4) |
| Gorna Trape | BS | BU | DA | 22 | 8 | 1 | Hap11(5)<br>Hap17(2) |
| Freundsheimer Weiher | BS | A | DA | | 3 | 2 | Hap25(1) |
| Wielenbach | BS | GER | DA | | 2 | 2 | |
| Kádárta | BS | HU | DA | 4 | 3 | 3 | |

| Population (site) | SB | Country | RC | Ms N | Seq N | Hap01 | Hap |
|---|---|---|---|---|---|---|---|
| Pét | BS | HU | DA | | 1 | | Hap09(1) |
| Schiopu | BS | RO | Mures/DA | | 1 | 1 | |
| Tebea | BS | RO | Cris/DA | | 1 | | Hap40(1) |
| Sohodol | BS | RO | Cris/DA | | 1 | | Hap40(1) |
| Racas | BS | RO | Cris/DA | | 1 | | Hap40(1) |
| Valea Boului | BS | RO | Cris/DA | | 1 | 1 | |
| Barcau | BS | RO | Cris/DA | | 2 | 1 | Hap40(1) |
| Valea Pestilor | BS | RO | Cris/DA | | 1 | | Hap39(1) |
| Valea Stoiaca | BS | RO | Cris/DA | | 2 | 1 | Hap40(1) |
| Nadas | BS | RO | Cris/DA | 13 | 12 | | |
| Derjana | BS | RO | Cris/DA | | 1 | | Hap40(1) |
| Clocotici | BS | RO | Caras/DA | 8 | 8 | | Hap36(1)<br>Hap37(7) |
| Natra | BS | RO | Caras/DA | | 1 | | Hap23(1) |
| Comarnic | BS | RO | Caras/DA | | 1 | | Hap23(1) |
| Toplita | BS | RO | Caras/DA | | 1 | | Hap23(1) |
| Ravistea | BS | RO | Caras/DA | | 8 | | Hap23(8) |
| Buhui | BS | RO | Caras/DA | | 2 | 2 | |
| Moravita | BS | RO | Caras/DA | | 5 | 1 | Hap19(1)<br>Hap23(3) |
| Dognecea | BS | RO | Caras/DA | | 2 | 1 | Hap23(1) |
| Ciornovãt | BS | RO | Caras/DA | 6 | 7 | | Hap22(6)<br>Hap23(1) |
| Caras | BS | RO | Caras/DA | | 1 | | Hap23(1) |
| Caianu | BS | RO | Caras/DA | | 1 | 1 | |
| Bacaia | BS | RO | Caras/DA | | 2 | 2 | |
| Balsa | BS | RO | Caras/DA | | 2 | 1 | Hap40(1) |
| Galben | BS | RO | Jiu/DA | | 1 | | Hap40(1) |
| Dragoiestilor | BS | RO | Jiu/DA | 22 | 5 | | Hap40(5) |
| Tamasesti | BS | RO | Mures/DA | | 4 | 4 | |
| Balsa | BS | RO | Mures/DA | | 4 | 3 | Hap40(1) |
| Cladovita | BS | RO | Mures/DA | | 4 | *2* | Hap10(2) |
| Carpan | BS | RO | Mures/DA | | 1 | | Hap34(1) |
| Geoagiu | BS | RO | Mures/DA | | 1 | 1 | |
| Conop | BS | RO | Mures/DA | | 3 | 1 | Hap40(2) |
| Valea Crisului | BS | RO | Mures/DA | | 4 | 4 | |
| Crisul | BS | RO | Mures/DA | | 3 | 3 | |
| Giacas | BS | RO | Mures/DA | | 2 | 2 | |
| Solocma | BS | RO | Mures/DA | | 3 | 3 | |
| Niraj | BS | RO | Mures/DA | | 1 | 1 | |
| Taraia | BS | RO | Olt/DA | | 1 | | Hap23(1) |
| Iazul | BS | RO | Olt/DA | | 1 | | Hap23(1) |
| Sinca | BS | RO | Olt/DA | | 1 | | Hap23(1) |
| Valea Adanca | BS | RO | Olt/DA | | 2 | 1 | Hap20(1) |
| Hartibaciu | BS | RO | Olt/DA | | 3 | | Hap23(1) |
| Venetia | BS | RO | Olt/DA | | 1 | | Hap23(1) |
| Simbrezi | BS | RO | Olt/DA | | 2 | | Hap23(2) |

| Population (site) | SB | Country | RC | Ms N | Seq N | Hap01 | Hap |
|---|---|---|---|---|---|---|---|
| Maierus | BS | RO | Olt/DA | | 1 | | Hap24(1) |
| Bogata | BS | RO | Olt/DA | | 1 | | Hap24(1) |
| Rupea | BS | RO | Olt/DA | | 4 | | Hap23(4) |
| Oituz | BS | RO | Siret/DA | | 1 | | Hap23(1) |
| Stramba | BS | RO | Siret/DA | | 1 | | Hap40(1) |
| Bichigiu | BS | RO | Somes/DA | | 1 | 1 | |
| Agrij | BS | RO | Somes/DA | | 1 | 1 | |
| Tetisu | BS | RO | Somes/DA | | 1 | 1 | |
| Poiana | BS | RO | Somes/DA | | 1 | 1 | |
| Sarasau | BS | RO | Tisa/DA | | 1 | 1 | |
| Valea Tejei | BS | RO | Tisa/DA | | 1 | 1 | |
| Valea Holita | BS | RO | Tisa/DA | | 1 | 1 | |
| Valea Mare | BS | RO | Tisa/DA | | 1 | 1 | |
| Vukovina Lake | BS | HR | Sava/DA | 6 | 5 | | Hap41(5) |
| Bačica Creek | BS | HR | Sava/DA | | 4 | | Hap42(3)<br>Hap44(1) |
| Bašnica River, Gračac | BS | HR | Sava/DA | | 2 | | Hap43(2) |
| Borovik Lake | BS | HR | Sava/DA | | 1 | | Hap43(1) |
| Dubočanka River | BS | HR | Sava/DA | | 1 | | Hap43(1) |
| Jaruga River, Stajničko polje | BS | HR | Sava/DA | | 2 | | Hap28(2) |
| Ježevo Lake, Velika Gorica | BS | HR | Sava/DA | | 1 | | Hap42(1) |
| Krapina River | BS | HR | Sava/DA | | 1 | | Hap43(1) |
| Mrežnica River, Generalski Stol | BS | HR | Sava/DA | | 2 | | Hap 27(2) |
| Plitvice Lakes - NP Plitvička jezera | BS | HR | Sava/DA | | 2 | | Hap28(2) |
| Pakra River, Kusonje | BS | HR | Sava/DA | | 1 | | Hap42(1) |
| Ribnjak Creek, Vladisovo | BS | HR | Sava/DA | | 3 | | Hap42(2)<br>Hap43(1) |
| Trećak Creek Staro Petrovo Selo | BS | HR | Sava/DA | | 2 | | Hap42(2) |
| Subocka River | BS | HR | Sava/DA | | 1 | | Hap42(1) |
| Šumetlica Creek | BS | HR | Sava/DA | | 3 | | Hap42(3) |
| Rakov Creek | AdS | HR | Pazinčica | | 1 | | Hap46(1) |
| Zeta River | AdS | MNE | Morača River | | 4 | | Hap28(1)<br>Hap29(1)<br>Hap42(2) |
| Liverovići Lake | AdS | MNE | ? | | 1 | | Hap42(1) |
| Paklenica River - NP Paklenica | AdS | HR | Paklenica River | | 2 | | Hap42(2) |
| Ričica River | AdS | HR | Ričica River | | 1 | | Hap45(1) |
| Unnamed stream Ferizaj (Uroševac) | AeS | KOS | ? | | 1 | | Hap26(1) |
| Zoo Zajak | NS | GER | Hatchery/RH | | 5 | 5 | |
| Farm in Belgium | NS | BE | Hatchery/ME | | 2 | 1 | Hap35(1) |
| Farm Oeversee | NS | GER | Hatchery/EI | | 5 | | Hap03(4)<br>Hap04(1) |

| Population (site) | SB | Country | RC | Ms N | Seq N | Hap01 | Hap |
|---|---|---|---|---|---|---|---|
| Farm Augsburg | BS | GER | Hatchery/DA | 24 | 15 | 8 | Hap06(3)<br>Hap07(1)<br>Hap08(1)<br>Hap32(2) |

Aquatic Invasions (2013) **8**

# *Crayfish plague agent detected in populations of the invasive North American crayfish Orconectes immunis (Hagen, 1870) in the Rhine River, Germany*

**Anne Schrimpf[1], Christoph Chucholl[2], Thomas Schmidt[1], Ralf Schulz[1]**

[1] University Koblenz-Landau, Institute for Environmental Sciences, Fortstrasse 7, 76829 Landau, Germany
[2] Fischereiforschungsstelle BW, Argenweg 50/1, 88085 Langenargen, Germany

## ABSTRACT

Crayfish plague, caused by the parasitic oomycete *Aphanomyces astaci*, has driven indigenous European crayfish species to regional extinction in many parts of Europe and is among the leading threats to the remaining populations. *A. astaci* is known to be carried by long-established invasive crayfish species of North American origin, which are also the main vectors of the plague pathogen. In this study, we examined whether a new invasive crayfish of North American origin, the calico crayfish (*Orconectes immunis*), also carries *A*. *astaci*. *Orconectes immunis* is a recent invader of the Upper Rhine plain, where it seems to displace its invasive predecessor *Orconectes limosus*, which is a known carrier of the agent of the crayfish plague. Using real-time PCR, we identified the calico crayfish as the *fourth* invasive crayfish species to be a carrier of the crayfish plague pathogen in Europe and we confirmed the infection with *A. astaci* in *O*. *limosus*. These findings support the concern that all North American crayfish species in European waters are carriers of the crayfish plague pathogen. Such knowledge should prove useful for conservation efforts, management, legislation, and public education about the spread of crayfish plague and non-indigenous crayfish species.

**Keywords**: Crayfish plague • *Aphanomyces astaci* • calico crayfish • crustacean disease • Rhine River • invasive American crayfish species • real-time PCR • pathogen vector

## INTRODUCTION

Invasive non-indigenous species are one of the leading threats to freshwater biodiversity, besides habitat deterioration (Sala et al. 2000; Gherardi 2007). All European freshwater crayfish species (Crustacea, Decapoda, Astacidae) are highly threatened by *Aphanomyces astaci* (Schikora 1906), an invasive, crayfish-specific parasite causing crayfish plague, i.e., a fatal disease (Söderhäll and Cerenius 1999). *Aphanomyces astaci* originates from North America and was probably first introduced to Europe in the late 1850s. Its natural hosts, North-American freshwater crayfish species, were not found during the first outbreaks of the disease in Europe but were repeatedly introduced later (Alderman 1996; Holdich et al. 2009). Out of a total of more than 460 crayfish species living in North America (Crandall and Buhay 2011), today at least eight species are established in Europe in the wild (Holdich et al. 2009; Chucholl and Pfeiffer 2010).

Although all North American crayfish species are suspected to be carriers of *A*. *astaci* (OIE 2009), only three of the North American crayfish species present in the wild in Europe have been shown to be carrier of the pathogen so far: the signal crayfish [*Pacifastacus leniusculus* (Dana, 1852)] (Unestam and Weiss 1970), the spiny-cheek crayfish [*Orconectes limosus* (Rafinesque, 1817)] (Vey et al. 1983), and the red swamp crayfish [*Procambarus clarkii* (Girard, 1852)] (Diéguez-Uribeondo and Söderhäll 1993). These three species belong to the 'Old' non-indigenous crayfish species in Europe, i.e. have been introduced into European waters before 1975 (summarized by Holdich et al. 2009).

One 'New' non-indigenous crayfish species in Europe is the North American calico crayfish [*Orconectes immunis* (Hagen, 1870)], which was first recorded at two locations in the Upper Rhine plain in the mid-1990s (Dussling and Hoffmann 1998; Dehus et al. 1999; Gelmar et al. 2006). The pathway of introduction of *O*. *immunis* is unclear; both an introduction from the pet trade and as fishing bait by Canadian soldiers had been suggested (Dehus et al. 1999; Gelmar et al. 2006). However, since the calico crayfish was not known in the German pet trade prior to its establishment in the Upper Rhine plain and because this species is popular as fishing bait in North America, an introduction as fishing bait seems more likely

(see Gelmar et al. 2006; Chucholl 2012b). Since its discovery, the calico crayfish has rapidly spread upstream and downstream in the Upper Rhine plain (Gelmar et al. 2006; Chucholl 2012a) and is currently colonizing a stretch of more than 98 km, where it inhabits a wide spectrum of habitat types (summarized in Chucholl 2012a; Figure 1). Despite its presence in Europe for almost two decades, its status as carrier of *A. astaci* is still unclear.

Calico crayfish seem to replace the formerly most abundant non-indigenous crayfish species of the Rhine River, the spiny-cheek crayfish (Gelmar et al. 2006; Chucholl et al. 2008), which has been present in the Upper Rhine catchment for at least five decades (Holdich et al. 2009). In laboratory experiments, calico crayfish were shown to be superior to spiny-cheek crayfish in direct aggressive interactions and competition for shelter (Chucholl et al. 2008). Furthermore, the calico crayfish exhibits a strongly *r*-selected life history: it is rather small (at most 50 mm in carapace length), features a high fecundity (up to 500 pleopodal eggs female$^{-1}$), and has the fastest recorded life cycle among the crayfish species present in Central Europe, combining a high growth rate and rapid maturation (within the first summer) with short longevity (2.5 years) (Chucholl 2012a).

Knowledge about the *A. astaci* carrier status of alien crayfish populations is imperative for native crayfish conservation, risk assessment and management strategies (Peay 2009). The aim of the present study was to evaluate the status of calico crayfish populations in the Rhine catchment as carrier of *A. astaci*.

## METHODS

To assess the *A. astaci* carrier status of calico crayfish in the Upper Rhine plain, we sampled two calico crayfish populations using traps and hand-held nets. One of the sampled populations was close to the currently known downstream invasion front of *O. immunis* (Germersheim, about 26 km north of Karlsruhe) and occurred in sympatry with *O. limosus*, whereas the second sampled population was located near the site of original introduction (Bühl, about 38 km south of Karlsruhe). The sampling site near the invasion front was located in the Rhine

River close to Germersheim (approx. Rhine km 390, 49°15'N, 8°25'E, see Figure 1) and the second sampling site was located with an aerial distance of about 63 km to the first sampling site at a small channel that is connected to the Rhine River near Bühl (8°04'N, 48°43'E). This site is in close proximity to the *O*. *immunis* occurrence reported by Dussling and Hoffmann (1998), i.e. one of the two locations where calico crayfish were first discovered in the mid-1990s. Calico crayfish is the exclusive crayfish species in this channel and previous to its establishment no crayfish had been known to occur there. In total, we collected 50 individuals of calico crayfish and 10 individuals of spiny-cheek crayfish from the Germersheim site in 2011 and 32 calico crayfish individuals from the Bühl site in 2012.

Upon capture, crayfish were identified using distinct features (Figure 2), and whole specimens were transported to the laboratory and frozen at -20°C. To evaluate the *A. astaci* carrier status we used the TaqMan® minor groove binder (MGB) real-time PCR (qPCR) according to Vrålstad et al. (2009). This method is the most specific and sensitive method to test for the presence of the crayfish plague pathogen (Tuffs and Oidmann 2011). Before DNA-extraction, we visually checked all calico crayfish for mechanical damage and melanisation. DNA was extracted from the soft abdominal cuticle, the inner joint of two walking legs, a part of the uropods and melanised spots when present using a CTAB-method as described in Vrålstad et al. (2009). The qPCR reaction was performed on a Mastercycler® ep realplex S (Eppendorf) using the TaqMan® Environmental Master Mix to avoid PCR inhibition (Strand et al. 2011). Differing from the published PCR program, the annealing temperature was increased to 62°C and the annealing time decreased to 15 seconds to further exclude possible false positive results (T. Vrålstad, personal communication). A negative control consisting of 5 µl nuclease free water was included together with a standard series of genomic DNA from a pure *A. astaci* culture. Data analysis was carried out using the software Real Plex 2.2 (Eppendorf).

The relative level of infection by the pathogen was based on the strength of the qPCR signal. The number of observed PCR-forming units in the reaction was assigned to semi-quantitative agent levels (according to Vrålstad et al. 2009).

Individuals with agent levels $A_0$ (no detection) and $A_1$ are considered uninfected, individuals with agent levels $A_2$ and higher are considered infected by *A. astaci* (see Table 1 caption for details).

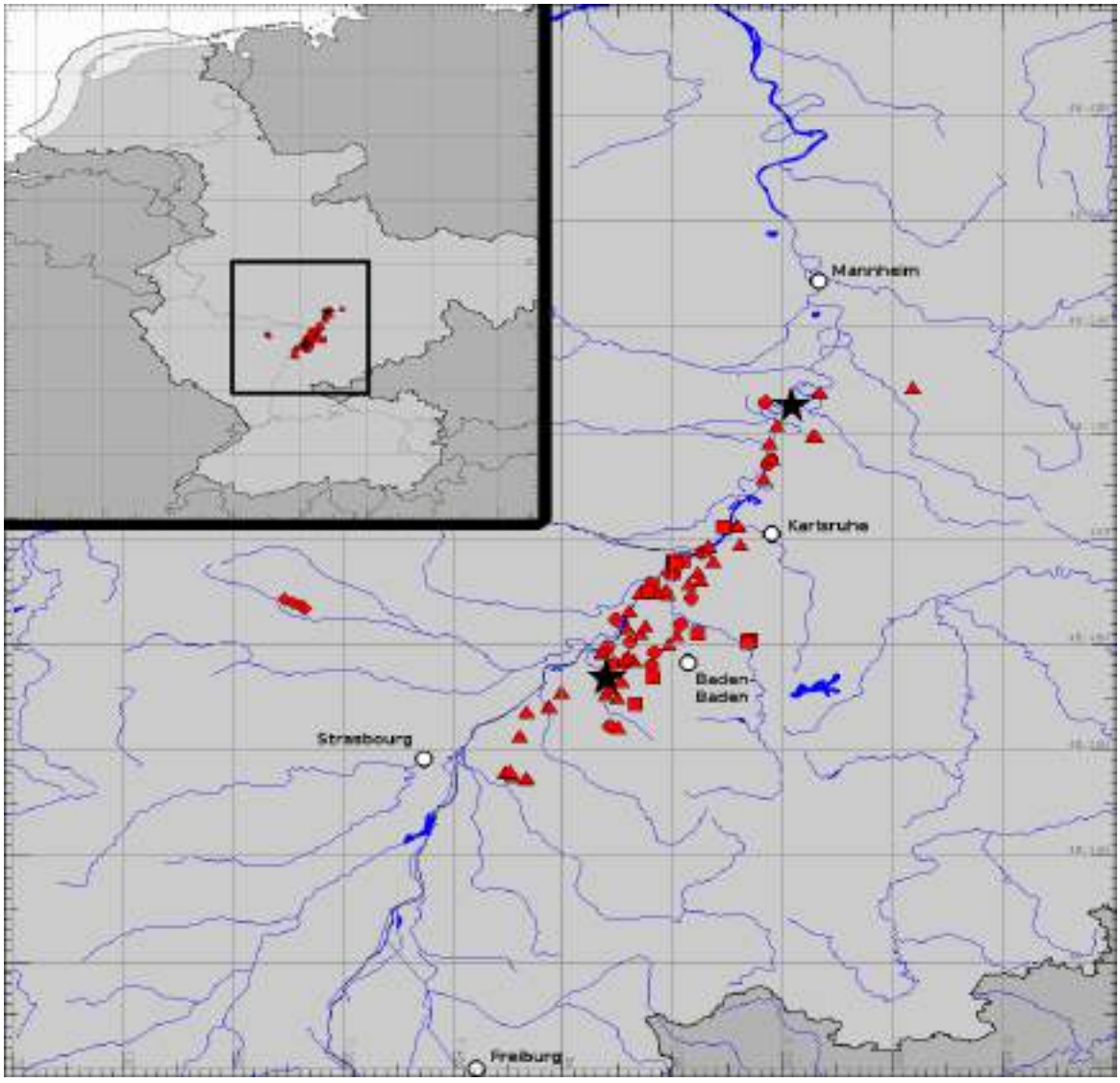


**Figure 1** The known distribution of *Orconectes immunis* in Europe as summarized in Chucholl (2012) with data from Chucholl & Dehus (2011; triangles), Collas et al. (2011; diamonds) and Gelmar et al. (2006; circles), completed with unpublished data from Chucholl (squares). Black stars indicate the sampling sites of this study. The international River Basin District Rhine (data: European Environment Agency, 2011) is colored light grey. Data of waters and national borders: GADM (2012).

## RESULTS

Using *A. astaci*-specific real-time PCR from a total of 92 crayfish a positive carrier status was found for about 60% of the tested crayfish (Table 1). At the sampling site Germersheim we found 23 positive detections out of 50 calico crayfish (46%) and six positive detections out of ten spiny-cheek crayfish (60%). Furthermore, *A. astaci* was detected in 26 of the 32 tested calico crayfish from Bühl (81%). Most individuals from both species and both sampling sites contained very low to moderate levels of agent DNA ($A_1$ to $A_4$). However, two calico crayfish from the Bühl site were infected at a very high level ($A_6$) and one spiny-cheek crayfish with mechanical damage from the Germersheim site was exceptionally high infected ($A_7$). Melanised areas were observed in eight calico crayfish from the Bühl site, in four calico crayfish from the Germersheim site, all of which were tested positive, and in one highly infected ($A_7$) spiny-cheek crayfish specimen.

**Table 1** Real-time PCR detection of *Aphanomyces astaci* in investigated specimens of *Orconectes immunis* and *Orconectes limosus* from the two sampling sites from the Upper Rhine plain in Germany and percentage of specimens detected positive. For each sampling site, number of analyzed crayfish, number and proportion of infected individuals, and relative level of infection according to Vrålstad et al. (2009) are given.

| Sampling site | Species | Crayfish (number) | Positives (number) | Positives (%) | Agent level[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $A_0$ | $A_1$ | $A_2$ | $A_3$ | $A_4$ | $A_5$ | $A_6$ | $A_7$ |
| Germersheim | *O. limosus* | 10 | 6 | 60 | 3 | 1 | | 3 | 2 | | | 1 |
| Germersheim | *O. immunis* | 50 | 23 | 46 | 21 | 6 | 13 | 9 | 1 | | | |
| Bühl | *O. immunis* | 32 | 26 | 81 | 3 | 3 | 4 | 18 | 2 | | 2 | |
| | **Total** | **92** | **55** | **60** | **27** | **10** | **17** | **30** | **5** | | **2** | **1** |

[a] Agent levels refer to semi-quantitative categories based on the numbers of observed PFUs ($PFU_{obs}$) from the *A. astaci*−specific real-time PCR. Agent level $A_0$: no detection; $A_1$: below the limit of detection ($PFU_{obs} < 5$); $A_2$: $5 \leq PFU_{obs} < 50$; $A_3$: $50 \leq PFU_{obs} < 10^3$; $A_4$: $10^3 \leq PFU_{obs} < 10^4$; $A_5$: $10^4 \leq PFU_{obs} < 10^5$; $A_6$: $10^5 \leq PFU_{obs} < 10^6$; $A_7$: $10^6 \leq PFU_{obs}$. $A_0$ and $A_1$ are both considered negative.

**DISCUSSION**

Using the currently most reliable molecular detection method for the agent of crayfish plague (Vrålstad et al. 2009; Tuffs and Oidtmann 2011), we have shown for the first time an *A*. *astaci* infection in calico crayfish. We found infections in this species at two sampling sites in the Upper Rhine plain with an aerial distance of about 63 km. Moreover, we confirmed the infection in spiny-cheek crayfish co-existing with calico crayfish. The relatively low agent levels ($\leq A_4$) of most infected crayfish are typical for North American crayfish species, which show an evolved defense reaction against *A*. *astaci* that normally prevents further spread of *A. astaci* hyphae within their body (Cerenius et al. 2003) and results in a latent infection. Thus, North American crayfish do usually not suffer from the disease but continuously release spores into the water with elevated spore levels prior to and during molting and mortalities (Strand et al 2012). In the present study we found one spiny-cheek crayfish with a physical injury. This injury may have stressed it´s immune system and thus explains the high infection status (A7) of this individual. The immune response can be visually observed in infected crayfish as melanised spots. However, melanised spots alone are not a good indicator for the infection status with *A. astaci* because they also appear as a reaction to physical injury. In the present study, melanisation was found in only 13 of the 29 positive tested crayfish. Therefore, the absence of melanised spots does not conclusively indicate the absence of an infection with *A*. *astaci*.

The positive verification of calico crayfish as carrier of the crayfish plague agent is worrying. Particularly, given the fast and successful spread of this 'New' non-indigenous crayfish species in the Upper Rhine plain. Moreover, the species replaces the 'Old' non-indigenous crayfish, spiny-cheek crayfish, from preferred habitats (Gelmar et al. 2006; Chucholl et al. 2008; Chucholl 2012a). Preliminary field observations suggest that calico crayfish inhabit a wider spectrum of habitats than spiny-cheek crayfish (Chucholl 2012a). Specifically, calico crayfish were found in shallow temporary backwaters adjacent to the Rhine River and brooks draining from the Schwarzwald, which are habitats from which spiny-cheek crayfish are typically absent. Calico crayfish might therefore spread *A. astaci* into habitats that were previously not colonized by spiny-cheek crayfish. However, to

date, calico crayfish have not come into contact with indigenous European crayfish species (Chucholl 2012a).

We assume that the calico crayfish will continue its fast invasion of the Rhine River and connected waterways. Artificial channels that connect the Rhine River to other large river catchment areas, such as the Danube, the Rhône, the Odra, and the Elbe, promote a fast spread of invasive aquatic species throughout Europe (Bij de Vaate et al. 2002) and will most likely also facilitate the further active spread of calico crayfish. In addition to active range expansion, secondary introductions, i.e. translocations of calico crayfish by humans, are also a potential mechanism of spread and have already occurred in Germany and possibly in France (Chucholl and Dehus 2011; Collas et al. 2011). Public and stakeholder information is therefore imperative to mitigate the risk of further secondary introductions, which are a major threat to otherwise isolated populations of indigenous European crayfish.

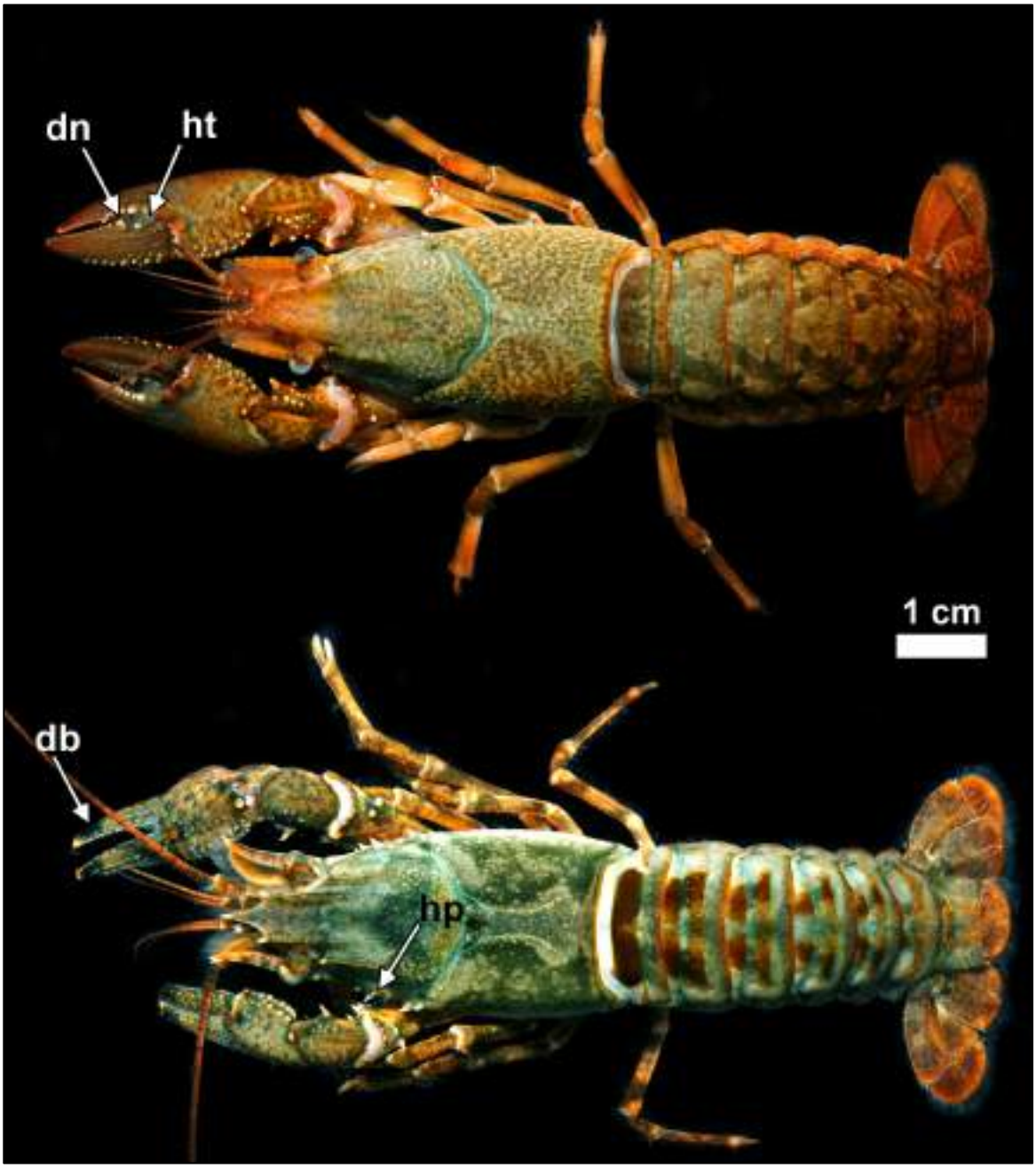


**Figure 2** *Orconectes immunis* ♀ (top) and *O*. *limosus* ♂ (bottom) from the Rhine River. Arrows denote key characters to distinguish the two species (modified from Gelmar et al. 2006 and Chucholl et al. 2008): dn – distinct tooth followed by a notch on the dactylus of the chelipeds (only present in *O*. *immunis*); ht – hair tufts on the ventral side of the chelae joints of the 1st and 2nd pereiopod (only present in *O*. *immunis*); db – distinct dark bandage adjacent to the orange cheliped tips (only present in *O*. *limosus*); hp – hepatic spines (only present in *O*. *limosus*).

Finally, it is important to note that there is accumulating evidence that different North American crayfish species are carriers of different strains of *A. astaci* (Huang et al. 1994, Kozubíková et al. 2011) and that these strains vary in their virulence (Jussila et al. 2011; Viljamaa-Dirks et al. 2011). An important question is therefore whether calico crayfish carry a different and possibly new *A. astaci* strain. This question is closely linked to the question of whether a) calico crayfish were already carriers of *A. astaci* when they were introduced to the Rhine catchment or whether b) calico crayfish were initially uninfected and got infected later, when they came into contact with *A. astaci*-carrying spiny-cheek crayfish. The positive verification of *A. astaci* in the single species population close to Bühl that has existed for about 14 years is a strong indication that calico crayfish were already carrier of the agent of crayfish plague when they were introduced to the Rhine catchment. In this case, calico crayfish may carry a new and possibly more virulent strain. However, we cannot rule out the possibility of recent human influence (e.g. by fishing gear that was contaminated with *A*. *astaci* spores) or an infection via animals (e.g. predatory fish feeding on infected crayfish; Oidtmann et al. 2002). Furthermore, *A. astaci* originating from spiny-cheek crayfish in the Rhine River could have reached the population by gradually infecting calico crayfish that are presumably distributed in the whole channel that connects the Rhine River with the sampling site in a stepping-stone manner. In the case of the Germersheim population *A. astaci* can be transmitted from calico crayfish to spiny–cheek crayfish and vice versa. We hope to resolve the question of the origin of *A. astaci* in European calico crayfish populations in the future, when methods become available that facilitate the assignment of *A*. *astaci* strains and the detection of new strains.

The positive verification of calico crayfish as carrier of *A. astaci* adds another species to the list of highly dangerous non-indigenous species. In particular, it adds another species to the list of *A. astaci*-carrying crayfish species. The result of this study has to be implemented in native crayfish conservation strategies. A site where calico crayfish is present has to be considered as a reservoir for *A. astaci*.

## ACKNOWLEDGMENTS

We would like to thank Jennifer Dahlem and Christian Löb for collecting samples and Gerd Mayer for his kind assistance during the photo session for Figure 2. Further, we thank four anonymous reviewers for comments on a previous version of the manuscript.

## REFERENCES

Alderman DJ (1996) Geographical spread of bacterial and fungal diseases of crustaceans. Revue Scientifique Et Technique - Office International Des Epizooties 15: 603–632

Bij de Vaate A, Jazdzewski K, Ketelaars HAM, Gollasch S, Van der Velde G (2002) Geographical patterns in range extension of Ponto-Caspian macroinvertebrate species in Europe. Canadian Journal of Fisheries and Aquatic Sciences 59: 1159-1174

Cerenius L, Bangyeekhun E, Keyser P, Söderhäll I, Söderhäll K (2003) Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*. Cellular Microbiology 5(5): 353–357

Chucholl C (2012a) Understanding invasion success: life-history traits and feeding habits of the alien crayfish *Orconectes immunis* (Decapoda, Astacida, Cambaridae). Knowledge and Management of Aquatic Ecosystems 404: 04

Chucholl C (2012b) Invaders for sale: trade and determinants of introduction of ornamental freshwater crayfish. Biological Invasions. doi: 10.1007/s10530-012-0273-2

Chucholl C, Dehus P. (2011) Flusskrebse in Baden-Württemberg, Fischereiforschungsstelle Baden-Württemberg (FFS), Langenargen, Germany, 92 pp.

Chucholl C, Pfeiffer M (2010) First evidence for an established Marmorkrebs (Decapoda, Astacida, Cambaridae) population in Southwestern Germany, in syntopic occurrence with *Orconectes limosus* (Rafinesque, 1817). Aquatic Invasions 5:405–412

Chucholl C, Stich HB, Maier G (2008) Aggressive interactions and competition for shelter between a recently introduced and an established invasive crayfish: *Orconectes immunis* vs. *O. limosus*. Fundamental and Applied Limnology Archiv für Hydrobiologie 172: 27-36

Collas M, Beinsteiner D, Fritsch S, Morelle S, L'Hospitalier M (2011) Première observation en France de l'Ecrevisse calicot, *Orconectes immunis* (Hagen, 1870). Annales scientifiques de la réserve de Biosphère transfrontalière Vosges du Nord - Pfälzerwald 16: 18 - 36

Crandall KA, Buhay JE (2011) Global diversity of crayfish (Astacidae, Cambaridae, and Parastacidae—Decapoda) in freshwater. Hydrobiologia 595: 295–301

Dehus P, Dussling U, and Hoffmann C (1999) Notes on the occurrence of the calico crayfish (*Orconectes immunis*) in Germany. Freshwater Crayfish 12: 786–790

Diéguez-Uribeondo J, Söderhäll K (1993) *Procambarus clarkii* Girard as a vector for the crayfish plague fungus, *Aphanomyces astaci* Schikora. Aquaculture and Fisheries Management 24: 761–765

Dussling U, Hoffmann C (1998) First discovery of a population of *Orconectes immunis* in Germany. Crayfish News 20(4): 5

European Environment Agency (2011) WISE River basin districts (RBDs)- version 1.4, Permalink 4CF96B9F-16D9-404B-A00A-BDD20A578E67

GADM (2012) GADM database of Global Administrative Areas Version 2.0, http://gadm.org/

Gelmar C, Pätzold F, Grabow K, Martens A (2006) Der Kalikokrebs *Orconectes immunis* am nördlichen Oberrhein: ein neuer amerikanischer Flusskrebs breitet sich rasch in Mitteleuropa aus (Crustacea: Cambaridae). Lauterbornia 56: 15–25

Gherardi F (Ed.), 2007. Biological invaders in inland waters: profiles, distribution and threats, Springer, Dordrecht

Holdich DM, Reynolds JD, Souty-Grosset C, Sibley PJ (2009) A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. Knowledge and Management of Aquatic Ecosystems 11: 394–395

Huang TS, Cerenius L, Söderhäll K (1994) Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci*, by random amplification of polymorphic DNA. Aquaculture 126: 1–9

Jussila J, Makkonen J, Vainikka A, Kortet R, Kokko H (2011) Latent crayfish plague (*Aphanomyces astaci*) infection in a robust wild noble crayfish (*Astacus astacus*) population. Aquaculture 321: 17–20

Kozubíková E, Viljamaa-Dirks S, Heinikainen S, Petrusek A (2011) Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague agent *Aphanomyces astaci*. Journal of Invertebrate Pathology 108: 214–216

Oidtmann B, Heitz E, Rogers D, Hoffmann RW (2002) Transmission of crayfish plague. Diseases of Aquatic Organisms 52(2): 159–167

OIE (2009) Crayfish plague (*Aphanomyces astaci*). Manual of Diagnostic Tests for Aquatic animals. Chapter 2.2.1. http://www.oie.int/fileadmin/Home/eng/health_standards/aahm/2010/2.2.01_CRAYFISH.pdf (Accessed 15 October 2012)

Peay S (2009) Selection criteria for "ark sites" for white-clawed crayfish. In: *Crayfish Conservation in the British Isles*. (Eds J. Brickland, D.M. Holdich, E.M. Imhoff), pp. 63–69. Leeds, UK, 25 March, 2009.

Sala OE, Chapin FS 3rd, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, Huber-Sannwald E, Huenneke L, Jackson RB, Kinzig A, Leemanns R, Lodge DM, Mooney HA, Oesterheld M, Poff NL, Sykes BH, Walker BH, Walker M, Wall DH (2000) Biodiversity scenario for the year 2100. Science 287: 1770–1774

Söderhäll K, Cerenius L (1999) The crayfish plague fungus: history and recent advances. Freshwater Crayfish 12: 11−35

Strand DA, Holst-Jensen A, Viljugrein H, Edvardsen B, Klaveness D, Jussila J, Vrålstad T (2011) Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. Diseases of Aquatic Organisms 95: 9–17

Strand DA, Jussila J, Viljamaa-Dirks S, Kokko H, Makkonen J, Holst-Jensen A, Viljugrein H, Vrålstad T (2012) Monitoring the spore dynamics of *Aphanomyces astaci* in the ambient water of latent carrier crayfish. Veterinary Microbiology **160:** 99-107

Tuffs S, Oidtmann B (2011) A comparative study of molecular diagnostic methods designed to detect the crayfish plague pathogen, *Aphanomyces astaci*. Veterinary Microbiology 153: 343–353

Unestam T, Weiss DW (1970) Host-parasite relationship between freshwater crayfish and the crayfish disease fungus, *Aphanomyces astaci*. Responses to infection by a susceptible and a resistant species. Journal of General Microbiol 60: 77–90

Vey A, Söderhäll K, Ajaxon R (1983) Susceptibility of *Orconectes limosis* Raff. to the crayfish plague, *Aphanomyces astaci* Schikora. Freshwater Crayfish 5: 284–291

Viljamaa-Dirks S, Heinikainen S, Nieminen M, Vennerström P, Pelkonen S (2011) Persistent infection by crayfish plague *Aphanomyces astaci* in a noble crayfish population – a case report. Bulletin of the European Association of Fish Pathologists 31(5): 182–188

Vrålstad T, Knutsen AK, Tengs T, Holst-Jensen A (2009) A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. Veterinary Microbiology 137: 146–155

Diseases of Aquatic Organisms (2012) **98**: 85-94.

# *Invasive crayfish and crayfish plague on the move: first detection of the plague agent Aphanomyces astaci in the Romanian Danube*

**Lucian Pârvulescu[1], Anne Schrimpf[2], Eva Kozubíková[3], Sara Cabanillas Resino[3], Trude Vrålstad[4,5], Adam Petrusek[3], Ralf Schulz[2]**

[1] West University of Timisoara, Faculty of Chemistry, Biology, Geography, Dept. of Biology and Chemistry, 300115 Timisoara, Romania
[2] University Koblenz-Landau, Institute for Environmental Sciences, 76829 Landau, Germany
[3] Charles University in Prague, Faculty of Science, Department of Ecology, 12844 Prague 2, Czech Republic
[4] Norwegian Veterinary Institute, Section of Mycology, 0033 Oslo, Norway
[5] University of Oslo, Department of Biology, 0316 Oslo, Norway

## ABSTRACT

Native European crayfish, such as *Astacus leptodactylus*, are threatened, among other factors, by the crayfish plague agent *Aphanomyces astaci*, dispersed by invasive North American crayfish. Two of these invaders, *Pacifastacus leniusculus* and *Orconectes limosus*, have extended their distribution in the River Danube catchment; the latter was detected for the first time in Romania in 2008. We monitored, at monthly intervals for over 2 yr, occurrence of native *A. leptodactylus* and invasive *O. limosus* at 6 sites on the Romanian Danube and checked for the invasive species in 4 of its tributaries. Between January 2009 and March 2011, the relative abundances of *O. limosus* steadily increased with time, while the native *A. leptodactylus* dramatically decreased in abundance. *O. limosus* expanded downstream at a rate of ca. 15 km $yr^{-1}$; in August 2011, it was already present in the upper 105 km of the Romanian Danube. An agent-specific real-time PCR analyses demonstrated the presence of *A. astaci* DNA in at least 32% of the analysed invasive (n = 71) and 41% of the native (n = 49) crayfish coexisting in the Danube. Furthermore, *A. astaci* was also detected in *A. leptodactylus* captured about 70 km downstream of the *O. limosus* invasion front (at the time of sampling). Assuming a steady rate of expansion, *O. limosus* may invade the sensitive Danube delta area in the mid-2060s, even without long-distance dispersal. The crayfish plague agent, however, may reach

the delta substantially earlier, through dispersal downstream among populations of native crayfish.

**Key words**: Crayfish plague • *Aphanomyces astaci* • Quantitative real-time PCR • Molecular diagnostics • Danube • Non-indigenous crayfish • *Orconectes limosus* • Indigenous crayfish • *Astacus leptodactylus*

## INTRODUCTION

Crayfish plague is a crayfish-specific disease caused by the oomycete *Aphanomyces astaci*, an obligate parasite of freshwater crayfish (Söderhäll & Cerenius 1999). While all European crayfish species are highly susceptible, infection does not usually cause disease outbreaks or death in North American crayfish species (OIE 2009) unless they are stressed (Persson & Söderhäll 1983, Cerenius et al. 1988) or exposed to extremely high concentrations of short-lived *A. astaci* zoospores (Diéguez-Uribeondo & Söderhäll 1993). It is assumed that *A. astaci* does not usually survive for more than a few weeks without a crayfish host (Söderhäll & Cerenius 1999, CEFAS 2000), and no durable stages (oospores) are known in the parasite's life cycle.

All 3 of the North American crayfish species most widely distributed in Europe - the spiny-cheek crayfish *Orconectes limosus* (introduced in 1890), the signal crayfish *Pacifastacus leniusculus*, and the red swamp crayfish *Procambarus clarkii* (both introduced in the 1950−60s) - act as carriers of the crayfish plague pathogen (e.g. Vey et al. 1983). They are, therefore, regional reservoirs of the disease in invaded areas and contribute to its further dispersion into areas dominated by native European crayfish species. However, the disease itself had already started to spread across the continent by the 1860s and 1870s (Alderman 1996), before the first docu- mented introductions of American crayfish species. There were repeated disease outbreaks in the late nineteenth and early twentieth centuries across a substantial part of the continent, leading to a sharp decline in native crayfish species. Regions affected by the disease were most of central and eastern Europe (including western Russia, Belarus, Ukraine, and the lower Danube basin), southern Fennoscandia, and parts of Western Europe, particularly Germany and the eastern half of France (Alderman 1996). Most

European regions not impacted by this particular major wave of crayfish plague outbreaks became affected later in the twentieth century, after intensive introductions of *P. leniusculus* and *P. clarkii* (e.g. Alderman et al. 1990, Taugbøl et al. 1993, Diéguez-Uribeondo 2006). Due to its virulence and devastating impact on indigenous European crayfish species, *Aphanomyces astaci* has been classified among the world's 100 worst invasive alien species (Lowe et al. 2004).

The only non-indigenous crayfish species in Romania, *Orconectes limosus*, was detected in the Romanian Danube for the first time in 2008 (Pârvulescu et al. 2009), having reached the area by downstream dispersal through the Danube. The species itself has been present in Europe since 1890, apparently due to a single successful introduction (Filipová et al. 2011). However, it did not colonise the Danube until 1985, when a wild population became established in Hungary (Puky & Schád 2006), from where it dispersed both upstream (e.g. Puky 2009) and downstream. Its downstream colonisation rate over 20 yr has been estimated at 13 to 16 km $yr^{-1}$ (Puky & Schád 2006). *O. limosus* has also entered Croatia through the Danube from Hungary; it has entered the River Drava and is spreading upstream at a rate of ~1.5 km $yr^{-1}$, affecting native populations of *Astacus leptodactylus* (Maguire & Klobuçar 2003, Faller et al. 2009).

The presence of *Orconectes limosus* in Romania may dramatically affect populations of the indigenous narrow-clawed crayfish *Astacus leptodactylus*, which occurs both in the Danube itself and in other watercourses of the catchment (Băcescu 1967). Furthermore, the indigenous stone crayfish *Austropotamobius torrentium* is common in most tributaries of the Romanian Danube (Pârvulescu & Petrescu 2010). These areas are also of crucial importance from a conservation perspective. Besides carrying *Aphanomyces astaci*, other characteristics of *O. limosus*, such as high fecundity and early maturation time, contribute to its negative pressure on native crayfish populations (Hamr 2002, Kozák et al. 2006, 2007, Schulz et al. 2006). Furthermore, a recently demonstrated potential for facultative parthenogenesis in this species (Buřič et al. 2011) may also contribute to its invasive potential.

The first outbreak of the crayfish plague in the upper Danube basin was registered in Bavaria, Germany, in early 1879; following this the outbreaks quickly

(within a few months) spread to upper Austria and further downstream (Alderman 1996). In less than 1 decade, the disease had spread as far as the Black Sea. Recently, crayfish plague outbreaks in the Danube catchment were reported from the Czech Republic (Kozubíková & Petrusek 2009) and Austria (Hochwimmer et al. 2009), and populations of *Orconectes limosus* in the Hungarian Danube have been confirmed to carry the crayfish plague pathogen (Kozubíková et al. 2010). Although Romania must have been substantially affected by outbreaks of this disease in the late nineteenth century (Alderman 1996), there are only a very few later reports of crayfish mass mortalities (for example, of native noble crayfish *Astacus astacus* in 1934−1935 in the Olt River, a tributary of the Danube; Bačescu 1967). However, their association with crayfish plague has never been definitively proven. During the last several years, molecular diagnostic tools employing conventional PCR and real-time PCR methods for the specific detection of *Aphanomyces astaci* have been developed, and these have accelerated and improved the detection and identification of the crayfish plague agent (Oidtmann et al. 2006, Hochwimmer et al. 2009, Vrålstad et al. 2009). The carrier status of symptom-free North American crayfish is also evaluated using these tools (Oidtmann et al. 2006, Kozubíková et al. 2009, 2011b, Vrålstad et al. 2009, 2011, Skov et al. 2011).

Reliable information on the presence of the crayfish plague agent in the Romanian Danube and on the level of threat posed to native crayfish by the quickly spreading *Orconectes limosus* is of key importance for freshwater management and species conservation actions. In this study, we monitored the distribution dynamics of *O. limosus* and *Astacus leptodactylus* in the upper Romanian Danube and its tributaries and analysed their carrier status using the most sensitive and reliable molecular diagnostic assays available. We employed the TaqMan® minor groove binder (MGB) real-time PCR (Vrålstad et al. 2009). Recent results show that this method offers higher sensitivity than any other presently available, and it is the preferred method for screening populations of North American crayfish for carrier status (Tuffs & Oidtmann 2011). Furthermore, this method leads to lower error rates in the detection of *Aphanomyces astaci* than alternatives based on conventional PCR (Kozubíková et al. 2011b).

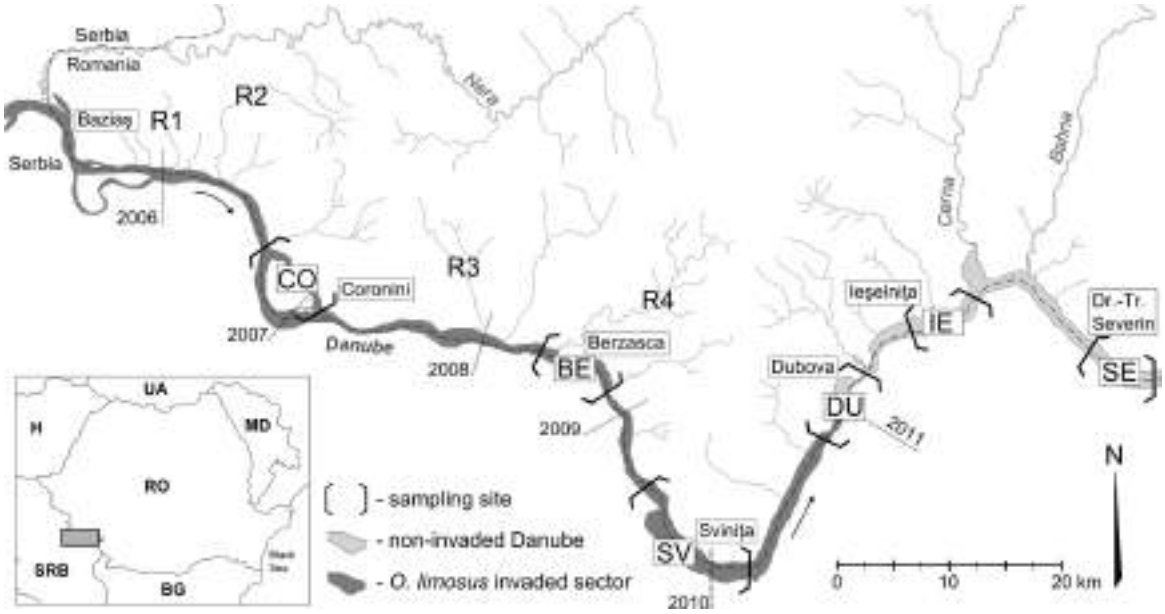


**Figure 1** Sampling sites in the River Danube (CO, BE, SV, DU, IE, SE) and its tributaries (R1 to R4; for river names see 'Materials and methods') in Romania and Serbia. Estimated years indicating the rate of invasion of this species are provided; *Orconetes limosus* was recorded at the DU site in August 2011. Dr.-Tr. Severin: Droberta-Turnu Severin. International license plate codes used for countries in inset map

## MATERIALS AND METHODS

### *Crayfish monitoring*

Six sites along the eastern section of the Romanian Danube, each covering 5 km of the river's shore stretch, were surveyed monthly, using the same capture effort, from January 2009 to March 2011 in order to estimate relative crayfish abundances. One surveyed site was the location of a well-known *Orconectes limosus* invasion, near the village of Coronini (CO in Fig. 1); and another site further downstream near the village of Berzasca (BE) had more recent evidence of invasion (Pârvulescu et al. 2009). In order to estimate the actual invasion front at Svinița, a (SV) properly, the investigated stretch of the river was twice as long (10 km) as for the other sites. Three sites downstream of the suspected invasion area were investigated: Dubova (DU), Ieşelniţa (IE) and Drobeta-Turnu Severin (SE). The downstream distance to each subsequent sampling site was approximately 15 km (Fig. 1).

Crayfish were captured using 2 bottom fishing nets of 1.5 m height, 25 m width, and a mesh size of ca. 30 mm, baited with fish meat and checked twice a week for a total period of 2 wk in each month. After capture, the species and sex of each individual was determined. Four Danube tributaries, Cameniţa River (R1 in Fig. 1), Radimna River (R2), Gornea River (R3) and Berzasca River (R4), were investigated using hand sampling of the riverbed during the summer of 2010 to assess whether *Orconectes limosus* shows a tendency to move upstream and colonise smaller watercourses. These tributaries are known to be populated by the stone crayfish *Austropotamobius torrentium* in areas between 1 to 10 km upstream of the confluence with the Danube (Pârvulescu & Petrescu 2010).

*Molecular diagnostics*

Crayfish were sacrificed at random to determine the presence of *Aphanomyces astaci* in both species by freezing them in separate plastic bags at −10°C. Altogether, we analysed 71 individuals of *Orconectes limosus* and 49 of *Astacus leptodactylus*. The crayfish were divided and analysed separately in 3 laboratories (in Oslo, Norway; Landau, Germany; and Prague, Czech Republic). For 42 crayfish (41 *O. limosus* and 1 *A. leptodactylus*) analysed in Prague, 50 mg of crayfish tissue, including the soft ventral abdominal cuticle and a part of the tail fan (uropods and/or telson), was dissected and processed according to the methodology described in Kozubíková et al. (2009). For the remaining 78 crayfish analysed in the laboratories at Oslo and Landau (30 *O. limosus* and 48 *A. leptodactylus*), 4 different tissue segments were dissected following methodology used by Vrålstad et al. (2011) and were preserved in 96% ethanol. The tissues included (1) the soft ventral abdominal cuticle; (2) the tail fan; (3) the inner joints of 2 walking legs; and (4) black spots (if present) on the exoskeleton. The melanised spots can be caused by an immune re- action against pathogens (including *A. astaci*) and may therefore indicate infection (Cerenius et al. 2003). The dissection tools were thoroughly disinfected after handling each individual crayfish.

DNA from the dissected tissues was extracted according to either Vrålstad et al. (2009; for the analyses in Oslo and Landau) or Kozubíková et al. (2009; for the

analyses in Prague). An environmental control (200 µl nuclease-free milliQ water in a tube, left open during the DNA extraction procedure) and an extraction blank control were included in the subsequent real-time PCR analyses as recommended by Vrålstad et al. (2009).

A quantitative TaqMan® MGB real-time PCR (Vrålstad et al. 2009) was conducted independently in all 3 laboratories to verify the presence of *Aphanomyces astaci*. This method is based on the detection of a 59 bp long *A. astaci*-specific fragment of the most variable part of the internal transcribed spacer region (ITS1) of the nuclear rDNA. Real-time PCR reactions were performed on a Mx3005P qPCR system (Stratagene) or on a Mastercycler® ep realplex S (Eppendorf) according to Strand et al. (2011), using the TaqMan® Environmental Master Mix to avoid PCR inhibition (Strand et al. 2011), and on an iQ5 Real-Time PCR Detection System (Bio-Rad) using the Universal PCR Master Mix (Life Technologies) according to Vrålstad et al. (2009). Data analysis was carried out using MxPro software version 4.10 (Stratagene), Real Plex 2.2 (Eppendorf), and iQ5 Optical System Software (Bio-Rad), respectively. A 4-fold dilution series of genomic DNA from a pure *A. astaci* culture (starting concentration: 5 ng $\mu l^{-1}$) served both as a standard and positive control. Unknown samples were included as concentrated (1×) and 5× diluted templates for the Stratagene and Eppendorf PCR setup, whilst concentrated (1×) and 10× diluted templates were analysed on the Bio-Rad system. Different dilutions, used to check for potential PCR-inhibition, were used to comply with standard protocols that had been established in the different laboratories.

The agent prevalence was quantified based on PCR-forming units (PFU), according to the methodology used by Vrålstad et al. (2009), in which 1 PFU corresponds to 1 amplifiable target DNA copy. Each sample was also assigned to semi-quantitative agent levels. Samples for which a weak signal below the limit of detection (= 5 PFU) was observed were considered negative (Vrålstad et al. 2009). Absolute quantification is possible in the absence of PCR inhibition above the limit of quantification (LOQ = 50 PFU according to Vrålstad et al. 2009). Possible real-time PCR inhibition was checked for by comparison of results obtained from concentrated and diluted samples. In the absence of inhibition, with

an acceptance of 15% variation, the difference in cycle threshold ($C_t$) values should be 2.32 ± 0.35 and 3.32 ± 0.48 for the 5 and 10× dilutions, respectively, when compared to undiluted DNA, (see Kozubíková et al. 2011b for more details). However, as many samples, particularly from *Orconectes limosus*, yielded results below LOQ, a quantitative comparison could not be made. Thus, we used a 1-tailed nonparametric Wilcoxon matched pairs test to evaluate whether the undiluted samples yielded significantly higher levels of agent DNA, which would at least imply that most samples were not substantially affected by inhibition.

The identity of *Aphanomyces astaci* was further confirmed by amplifying and sequencing a 529 bp long ITS fragment (including ITS1 as well as ITS2) from 6 DNA isolates representing both host crayfish species, according to protocol used by Oidtmann et al. (2006), with primers 42 and 640 designed to be specific for *A. astaci* (but see Kozubíková et al. 2011b for discussion on their limitation). The PCR products were sequenced in the forward direction using the primer 42, and resulting sequences (GenBank accession numbers JN713915−JN713917) were aligned in Mega 5 (Tamura et al. 2011) together with those representing all presently known *A. astaci* genotype groups (As, PsI, PsII, Pc, Or) isolated from various crayfish hosts: *Astacus astacus*, *Pacifastacus leniusculus*, *Procambarus clarkii*, as well as *Orconectes limosus* (see Kozubíková et al. 2011a). The alignment was then checked visually for the presence of differences among sequences.

## RESULTS

*Occurrence and dynamics of Orconectes limosus and Astacus leptodactylus*

The front of the downstream invasion of the River Danube by *Orconectes limosus* was, by the end of the regular monitoring (March 2011), found to be near Svinița, 998 km upstream from the Black Sea (Fig. 1). The species was first detected at this site in May 2010, when only the upper 5 km stretch of Site SV contained *O. limosus*, whereas no individuals of the invasive species were captured in the downstream 5 km stretch. During the 2 yr duration of the regular monitoring, *O. limosus* moved down the River Danube by about 21 km from below Site BE to Site SV (Fig. 1). However, the most recent investigation, in August 2011, has confirmed the species at Dubova (Site DU), 25 km further downstream of Sviniţa.

Altogether, 2068 individuals of *Orconectes limosus* and 4552 of *Astacus leptodactylus* were captured during the survey period (January 2009 to March 2011). The 3 downstream sites in the River Danube (DU, IE, and SE) contained only *A. leptodactylus*, of which 3006 specimens were collected during the present study. A comparison of the relative abundances of both crayfish species at the upper 3 sampling sites along the Danube (CO, BE, and SV) shows a very large decline in the native species *A. leptodactylus* at Site BE, at which the relative abundance of the invasive species was about 75% (Fig. 2). The relative abundance of *O. limosus* at the invasion front (SV) in early 2011 was about 7.2% (Fig. 2). The sex ratio of *O. limosus* varied among sites: the percentage of captured males was 60.1% at CO, and decreased further downstream to 59.4 % at BE and 47.1 % at SV.

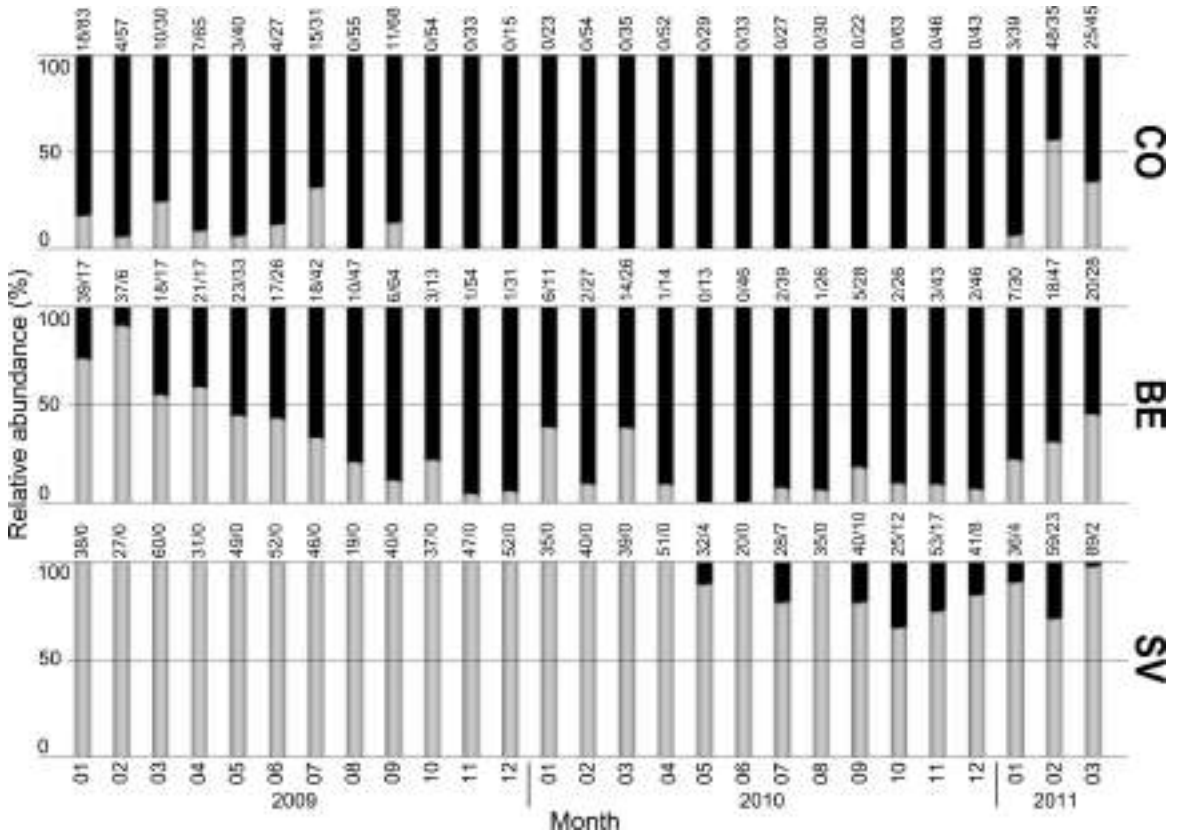


**Figure 2** *Astacus leptodactylus* and *Orconectes limosus*. Relative abundance of native *A. leptodactylus* (grey bars) and invasive *O. limosus* (black bars) at 3 sites of the River Danube (see Fig. 1) over a period of 27 mo. Upper graph: CO (Coronini); middle graph: BE (Berzasca); lower graph: SV (Svinița; invasion front). Numbers above the bars refer to the absolute total number of *A. leptodactylus* and *O. limosus*, respectively, caught during each sampling period

The crayfish monitoring results differed between tributaries, depending mainly on the width of the tributary. In the small tributaries, Cameniţa, Radimna and Gornea (R1 to R3 in Fig. 1), each of which is less than 3 m wide at the confluence with the Danube, the invasive species was only found directly at the confluence, and only extended for some tens of metres upstream. In the Berzasca tributary (R4), with a mean width of about 7 m at the confluence, the invasive species was found up to 1400 m upstream of the tributary mouth. This was despite the fact that the time available for colonising this tributary was much shorter than for the tributaries R1 to R3 situated further upstream along the Danube. During the investigation period, a total of 161 individuals of *Orconectes limosus* were captured in the Berzasca. The distance separating the invasive species and the native *Austropotamobius torrentium* populations in the tributaries

was 1050 m at Cameniţa, 3200 m at Radimna, 4550 m at Gornea and 10800 m at Berzasca.

*Molecular detection of Aphanomyces astaci*

The crayfish plague agent *Aphanomyces astaci* was detected in 32% of the investigated *Orconectes limosus* specimens collected from Sites CO and BE (Table 1). PFU values in undiluted DNA samples from *A. astaci*-positive individuals ranged between 5 and approximately 27 000, corresponding to agent levels from A2 to A5 (Table 2), according to Vrålstad et al. (2009). Most of these samples were found to contain a low amount of the target DNA (Levels A2 and A3), and only one showed a high agent level (A5). Furthermore, *A. astaci* was also detected in 20 of the 49 investigated *Astacus leptodactylus* specimens (Table 2), even though their general state and behaviour did not indicate acute crayfish plague. Most importantly, we also detected *A. astaci* in 3 individuals of *A. leptodactylus* from Site SE, situated 68 km downstream of the *O. limosus* invasion front known at the time of sampling. All 6 ITS sequences obtained from isolates from 5 *O. limosus* from both Sites CO and BE, and 1 *A. leptodactylus* from BE, were invariant. They corresponded exactly to sequences from *A. astaci* strains of all 5 known genotype groups of the pathogen, including the one obtained from *O. limosus* (Kozubíková et al. 2011a). The sequencing thus corroborated the results of the real-time PCR analysis.

**Table 1** *Aphanomyces astaci* infecting *Astacus leptodactylus* and *Orconectes limosus*. Number of crayfish from the Romanian Danube analysed for *A. astaci* infection using real-time PCR and percentage of specimens detected positive

| Locality | Species | No. of analysed crayfish | *A. astaci*-positive | % infected |
|---|---|---|---|---|
| Coronini (CO) | *A. leptodactylus* | 1 | 1 | 100 |
| | *O. limosus* | 37 | 9 | 24 |
| Berzasca (BE) | *A. leptodactylus* | 24 | 6 | 25 |
| | *O. limosus* | 34 | 14 | 41 |
| Svinița (SV) | *A. leptodactylus* | 21 | 10 | 48 |
| Severin (SE) | *A. leptodactylus* | 3 | 3 | 100 |
| Total | *A. leptodactylus* | 49 | 20 | 41 |
| | *O. limosus* | 71 | 23 | 32 |

**Table 2** *Aphanomyces astaci* infecting *Astacus leptodactylus* and *Orconectes limosus*. Summary data on the real-time PCR detection of *A. astaci* from native and invasive crayfish from 4 sites on the River Danube (see Fig. 1). Agent levels refer to semi-quantitative categories based on the observed numbers of PCR-forming units ($PFU_{obs}$), according to Vrålstad et al. (2009). Agent Level 0 ($A_0$; no detection) and Agent Level 1 ($A_1$; detection below the limit of detection: $PFU_{obs} < 5$ PFU) are both considered negative. Remaining categories ($A_2$ to $A_5$) are considered as a positive detection of the pathogen. $A_2$: 5 PFU ≤ $PFU_{obs}$ < 50 PFU, $A_3$: 50 ≤ $PFU_{obs}$ < $10^3$ PFU, $A_4$: $10^3$ PFU ≤ $PFU_{obs}$ < $10^4$ PFU, and $A_5$: $10^4$ PFU ≤ $PFU_{obs}$ < $10^5$ PFU

| Sampling site | Species | No. of crayfish analysed | Agent level | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $A_0$ | $A_1$ | $A_2$ | $A_3$ | $A_4$ | $A_5$ |
| Coronini (CO) | *A. leptodactylus* | 1 | | | | | | 1 |
| | *O. limosus* | 37 | 21 | 7 | 4 | 4 | | 1 |
| Berzasca (BE) | *A. leptodactylus* | 24 | 18 | | 2 | 3 | | 1 |
| | *O. limosus* | 34 | 13 | 7 | 10 | 4 | | |
| Svinița (SV) | *A. leptodactylus* | 21 | 11 | | 5 | 2 | 3 | |
| Severin (SE) | *A. leptodactylus* | 3 | | | | 3 | | |

Significantly higher levels of *Aphanomyces astaci* DNA were found in samples of undiluted template DNA in the PCR setup, in comparison to 5× diluted template samples using Environmental Master Mix (1-tailed Wilcoxon matched pairs test, n = 31, Z = 2.86, p = 0.002) as well as when compared to 10× diluted samples using Universal PCR Master Mix (n = 9; Z = 2.67; p = 0.004). However, for 1 *Astacus leptodactylus* and 2 *Orconectes limosus* individuals, *A. astaci* was detected in diluted samples only. This suggests that although inhibition was not a major problem, it occasionally affected the efficiency of detection, potentially underestimating the extent of infection.

## DISCUSSION

Our study clearly showed that *Orconectes limosus* is rapidly spreading downstream in the Romanian stretch of the Danube, and that its populations are infected by the crayfish plague pathogen *Aphanomyces astaci*. We also demonstrated that *A. astaci* had been transferred to local populations of the native narrow-clawed crayfish *Astacus leptodactylus*, which strongly declined in coexistence with *O. limosus*. Furthermore, the pathogen was detected in an *A. leptodactylus* population well in advance of the main invasion front of the invasive

species. The rate of *O. limosus* invasion in the Romanian Danube identified by our study was comparable to other studies quantifying its dispersal in this river. Given that in January 2009 *O. limosus* was found in the Berzasca region (Site BE; 1015 km before the Danube flows into the Black Sea), and reached Sviniţa by May 2010 (Site SE; 998 km upstream of the Black Sea), the estimated average colonisation speed is 1.23 km $mo^{-1}$, or about 15 km $yr^{-1}$, consistent with the findings of other studies (Puky & Schád 2006, Hudina et al. 2009). In August 2011 the species reached Dubova (DU), having moved 25 km down stream of Sviniţa, at a rate of 1.56 km $mo^{-1}$, slightly above the average calculated colonisation speed.

The serious decline in the relative abundance of the indigenous species *Astacus leptodactylus* in the Coronini area (CO), and the decrease in its relative abundance in the Berzasca area (BE), suggests that the invasive species has a tendency to eliminate the indigenous populations. The population dynamics of native European crayfish susceptible to crayfish plague that are in contact with invasive crayfish populations is, amongst other aspects, strongly dependent on the presence of the pathogen. For example, coexistence and a slow displacement of the native species due to the competitive advantages of the invader has been observed for mixed populations of *Orconectes limosus* and *Astacus astacus* in which the pathogen was not detected (Schulz et al. 2006) and also for coexisting *A. astacus* and presumably non-infected *Pacifastacus leniusculus* (Westman et al. 2002). When *Aphanomyces astaci* is present, however, a fast elimination of whole populations of European native crayfish can be expected (Westman et al. 2002).

Our confirmation of the causative agent of the crayfish plague in the Romanian Danube highlights the threat that the presence of *Orconectes limosus* poses to the indigenous crayfish populations (Longshaw 2011). We confirmed that substantial proportions of *O. limosus* carry the crayfish plague pathogen in both stretches of the Danube we investigated, where the species is already well established (CO and BE). Since the abundance of *O. limosus* is still low at the present invasion front, no conclusions can yet be made on the presence of *Aphanomyces astaci* for this site. However, we provided real-time PCR-based evidence for the presence of *A. astaci* in native *Astacus leptodactylus* (with

melanised spots on the exoskeleton) about 70 km below the suspected invasion front of *O. limosus* at the time of sampling. Although we cannot rule out that some infected *O. limosus* could have dispersed substantially farther than suggested by our monitoring data, it is likely that *A. astaci* is spreading downstream ahead of the invasive crayfish, by gradual expansion through local *A. leptodactylus* populations. Multiple mechanisms may contribute to the pathogen dispersal, apart from movement activity of crayfish. These include, for example, passive transport of infected live or dead crayfish (or even their exuviae). Furthermore, a direct dispersal of zoospores carried for a short distance by river currents may also be a source of infection for populations of *A. leptodactylus* further downstream. Due to these mechanisms, *A. astaci* may move down the River Danube much faster than the invasion front of *O. limosus*.

Dispersal of the crayfish plague through a river with abundant populations of susceptible crayfish may be very fast, as demonstrated by the rate at which the Danube catchment was affected during the major outbreak of crayfish plague in the 1870s−1890s (Alderman 1996). It is, therefore, possible that the pathogen will reach the Danube delta within a few years. On the other hand, if the rate of expansion of *Orconectes limosus* does not change substantially, the invasive crayfish itself may reach the Danube delta in the mid-2060s. However, anthropogenic long-range dispersal (for example, by shipping) may speed up its invasion considerably. The importance of invasive crayfish for introduction and dispersal of *Aphanomyces astaci* has also recently been reported for *Procambarus clarkii* in Italy (Aquiloni et al. 2011).

In crayfish populations sensitive to crayfish plague, the pathogen prevalence usually gradually increases over time, mostly reaching 100% (OIE 2009). This typically results in complete elimination of populations impacted by the plague, including stream populations of *Austropotamobius torrentium* (e.g. Kozubíková et al. 2008). However, some evidence exists that *Astacus leptodactylus* is at least sometimes more resistant to infection than other European species (Unestam 1969) and may be able to coexist with *Aphanomyces astaci* for extended periods of time. This has been implied particularly for some Turkish lakes where the pathogen has been assumed to be present continuously for over 2 decades,

despite the absence of American crayfish species in the country (Harlioğlu 2008). Recent molecular analyses of material from one of these Turkish lakes (Svoboda et al. 2012) confirmed that *A. astaci* may indeed persist in a population of *A. leptodactylus* that retains sufficient density for commercially harvesting. It is, therefore, possible that *A. leptodactylus* populations in the Danube will not be completely eliminated after introduction of *A. astaci* but will rather serve, at least temporarily, as a potential source of its further dispersal. This is also supported by our finding of *A. leptodactylus* in winter 2011 at Site CO, in which *Orconectes limosus*, and supposedly also *A. astaci*, had been present for about 4 yr (Fig. 2).

The facts that not only American but also native species of crayfish may serve as the source of crayfish plague infection and that not all North American crayfish are carriers of the pathogen, together with observed coexistence between European and North American crayfish species (Nylund & Westman 1992, Westman et al. 2002, Hudina et al. 2009, Skov et al. 2011), highlight the importance of using reliable detection methods for testing for presence of *Aphanomyces astaci* in suspected host populations. In the present study, we successfully applied MGB real-time PCR as proposed by Vrålstad et al. (2009) and Strand et al. (2011) to evaluate infection status independently in 3 laboratories and used ITS sequencing as an independent verification of the crayfish plague agent *A. astaci*.

We mostly found relatively low PFU values and corresponding very low (A2) and low (A3) agent levels (according to Vrålstad et al. 2009) but, nevertheless, unambiguously confirmed the presence of *A. astaci* in both invasive *Orconectes limosus* and native *Astacus leptodactylus*. Our results suggest that PCR inhibition was unlikely a major problem in our analyses. Nonetheless, the proportion of infected individuals might be underestimated, as we could not analyse the whole crayfish body for *A. astaci* presence, and detections of trace amounts of pathogen DNA (under 5 PFU) were considered negative to avoid potential false positives.

The results of monitoring of Danube tributaries confirmed the observations of Petrusek et al. (2006) from the Elbe catchment, where *Orconectes limosus* rarely penetrates far into loworder watercourses unless aided by humans. However, the risk of the crayfish plague agent expanding to native crayfish populations in small

streams cannot be ignored, even in the absence of any direct encounters with *O. limosus*, mainly because uncontrolled fishing takes place regularly in this area. *Aphanomyces astaci* can be transferred from one water body to the next through fishing gear, contaminated traps, the transfer of infected animals, and probably even by predators preying on crayfish (Nylund & Westman 1992, Alderman 1996, Oidtmann et al. 2002). It has been estimated that if the European-wide distribution and abundance of native stocks continue to decline and invasive crayfish species continue rapid expansion at the present rates, all European watersheds suitable for crayfish might be inhabited by invasive species within 100 yr (Skurdal & Taugbøl 2002, Holdich et al. 2009). The example of Sweden, where only around 5% of the native crayfish populations remain since the introduction of the crayfish plague (Fjälling & Fürst 1988, Bohman et al. 2006), shows the need for increased conservation effort to protect native species as soon as the plague agent enters a country.

Since invasive crayfish are not as widespread in Romania as in most other European countries, Romanian conservation managers have the opportunity to learn from the experience of other countries and act before it is too late. Based on our data, we assume the crayfish plague agent may reach the Danube delta very soon, and no measures can stop or slow it down. Protection of Romanian native crayfish populations may be made more efficient in preservation of *Astacus astacus* and *Austropotamobius torrentium* by identifying or artificially establishing so-called 'ark-sites' (Peay 2009a). Detailed investigations, and a well-organised management plan based on sound research findings, are strongly recommended, but additional conservation measures including education of local stakeholders and communities, fishermen, and children (Peay 2009b) are needed as well. This is particularly relevant in this region, where fishing is a major occupation, and simple and inexpensive measures (such as avoidance of using the same fishing tools in other rivers as in the Danube) may prevent the disease spreading from the Danube into the tributaries.

**ACKNOWLEDGEMENTS**

We thank the following for the approval of our study: the Romanian Academy (2257/CJ/2009), Iron Gates (Por ile de Fier) Natural Park Administration (136/2010; 3847/2011), the Environmental Protection Agency, Caraş-Severin County (423/2010; 1884/2011), and Mehedinţi County (1404/2010; 2785/2011). Some of the analyses were funded by the Czech Ministry of Education (project no. MSM0021620828), the Grant Agency of the Charles University (154110), and the Czech Science Foundation (206/08/H049). The field investigations were supported by the Bio-Team Association (Timisoara, Romania). We thank Cristian Paloş, Daniel Vuia, Amelia Ţundrea, and Dorin Ianoş for help with the fieldwork, and David Strand for assistance in the laboratory. Three anonymous reviewers provided useful comments on a previous version of the manuscript.

**LITERATURE CITED**

Alderman DJ (1996) Geographical spread of bacterial and fungal diseases of crustaceans. Rev Sci Tech Off Int Epizoot 15:603−632

Alderman DJ, Holdich D, Reeve I (1990) Signal crayfish as vectors in crayfish plague in Britain. Aquaculture 86:3−6 Aquiloni L, Martin MP, Gherardi F, Diéguez-Uribeondo J (2011) The north American crayfish Procambarus clarkii is the carrier of the oomycete *Aphanomyces astaci* inItaly. Biol Invasions 13:359−367

Băcescu MC (1967) Fauna Republicii Socialiste România, Vol 4. Crustacea: Decapoda (The fauna of Romanian Socialist Republic, Vol 4. Crustacea, Decapoda). Academic Publishing House, Bucharest (in Romanian)

Bohman P, Nordwall F, Edsman L (2006) The effect of the large-scale introduction of signal crayfish on the spread of crayfish plague in Sweden. Bull Fr Peche Piscicult 380−381:1291−1302

Buřič M, Hulák M, Kouba A, Petrusek A, Kozák P (2011) A successful crayfish invader is capable of facultative parthenogenesis: a novel reproductive mode in decapod crustaceans. PLoS ONE 6:e20281

CEFAS (Centre for Environment Fisheries and Aquaculture Science) (2000) Summary final report: effects of exposure to high and low temperatures on the survival of the cray- fish plague fungus *A. astaci* in vitro and in vivo. Australian Quarantine and Inspection Service, Canberra

Cerenius L, Söderhäll K, Persson M, Axajon R (1988) The crayfish plague fungus *Aphanomyces astaci* diagnosis, isolation, and pathobiology. Freshw Crayfish 7:131−144

Cerenius L, Bangyeekhun E, Keyser P, Söderhäll I, Söderhäll K (2003) Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*. Cell Microbiol 5:353−357

Diéguez-Uribeondo J (2006) Pathogens, parasites and ectocommensals. In: Souty-Grosset C, Holdich DM, Noël PY, Reynolds JD, Haffner P (eds) Atlas of crayfish in Europe. Patrimoines Naturels 64, Muséum National d'Histoire Naturelle, Paris, p 131−149

Diéguez-Uribeondo J, Söderhäll K (1993) Procambarus clarkii Girard as a vector for the crayfish plague fungus, *Aphanomyces astaci* Schikora. Aquacult Res 24:761−765

Faller M, Hudina S, Klobučar G, Maguire I (2009). Spread of invasive spiny-cheek crayfish (*Orconectes limosus*) in Croatia: filling knowledge gaps and identifying confinement measures. In: Kozák P, Kouba A (eds) Abstract book. Future of native crayfish in Europe. Regional European Crayfish Workshop: 7th−10th September 2009, Písek. Research Institute of Fish Cultures and Hydrobiology, Vodnany

Filipová L, Lieb DA, Grandjean F, Petrusek A (2011) Haplotype variation in the spiny-cheek crayfish *Orconectes limosus*: colonization of Europe and genetic diversity of native stocks. J N Am Benthol Soc 30:871−881

Fjälling A, Fürst M (1988) The development of a fishery for the crayfish *Pacifastacus leniusculus* in Sweden 1960−86. Freshw Crayfish 7:223−230

Hamr P (2002) Orconectes. In: Holdich DM (ed) Biology of freshwater crayfish. Blackwell Science, Oxford, p 585−608

Harlioğlu MM (2008) The harvest of the freshwater crayfish *Astacus leptodactylus* Eschscholtz in Turkey: harvest history, impact of crayfish plague, and present distribution of harvested populations. Aquacult Int 16:351−360

Hochwimmer G, Tober R, Bibars-Reiter R, Licek E, Steinborn R (2009) Identification of two GH18 chitinase family genes and their use as targets for detection of the crayfishplague oomycete *Aphanomyces astaci*. BMC Microbiol 9:184

Holdich DM, Reynolds JD, Souty-Grosset C, Sibley PJ (2009) A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. Knowl Manag Aquat Ecosyst 394−395:11

Hudina S, Faller M, Lucić A, Klobučar G, Maguire I (2009) Distribution and dispersal of two invasive crayfish species in the Drava River basin, Croatia. Knowl Manag Aquat Ecosyst 394−395:9

Kozák P, Buřič M, Policar T (2006) The fecundity, time of egg development and juvenile production in spiny-cheek crayfish (*Orconectes limosus*) under controlled conditions. Bull Fr Peche Piscicult 380−381:1171−1182

Kozák P, Hulák M, Policar T, Tichý F (2007) Studies of annual gonadal development and gonadal ultrastructure in spiny-cheek crayfish (*Orconectes limosus*). Bull Fr Peche Piscicult 384:15−26

Kozubíková E, Petrusek A (2009) Račí mor – přehled dosavadních poznatků o závažném onemocnění raků a zhodnocení situace v České republice. [Crayfish plague – review of present knowledge on serious disease of crayfish and evaluation of the situation in the Czech Republic]. Bulletin VÚRH Vodňany 45(2–3):34–57

Kozubíková E, Petrusek A, Ďuriš Z, Martín MP, Diéguez-Uribeondo J, Oidtmann B (2008) The old menace is back: recent crayfish plague outbreaks in the Czech Republic. Aquaculture 274:208–217

Kozubíková E, Filipová L, Kozák P, Ďuriš Z, Martín MP, Diéguez-Uribeondo J, Oidtmann B, Petrusek A (2009) Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in invasive American crayfishes in the Czech Republic. Conserv Biol 23:1204–1213

Kozubíková E, Puky M, Kiszely P, Petrusek A (2010) Crayfish plague pathogen in invasive North American crayfish species in Hungary. J Fish Dis 33:925−929

Kozubíková E, Viljamaa-Dirks S, Heinikainen S, Petrusek A (2011a) Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague agent *Aphano- myces astaci*. J Invertebr Pathol 108:214−216

Kozubíková E, Vrålstad T, Filipová L, Petrusek A (2011b) Re-examination of the prevalence of *Aphanomyces astaci* in North American crayfish populations in Central Europe by TaqMan MGB real-time PCR. Dis Aquat Org 97:113−125

Longshaw M (2011) Diseases of crayfish: a review. J Invertebr Pathol 106:54−70

Lowe S, Browne M, Boudjelas S, De Poorter M (2004) 100 of the world's worst invasive alien species. A selection from the Global Invasive Species Database, the Invasive Species Specialist Group (ISSG), a specialist group of the Species Survival Commission (SSC) of the IUCN. IUCN, Gland

Maguire I, Klobučar G (2003) Appearance of *Orconectes limosus* in Croatia. Crayfish News. IAA Newsl 25:7

Nylund V, Westman K (1992) Crayfish diseases and their control in Finland. Report from the EIFAC workshop on crayfish management and stocking, 22−23 August 1991, Kuopio, Finland. Finn Fish Res 14:107−118

Oidtmann B, Heitz E, Rogers D, Hoffmann RW (2002) Transmission of crayfish plague. Dis Aquat Org 52:159−167

Oidtmann B, Geiger S, Steinbauer P, Culas A, Hoffmann RW (2006) Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction. Dis Aquat Org 72:53−64

OIE (Office international des épizooties) (2009) Crayfish plague (*Aphanomyces astaci*). Chapter 2.2.1. In: Manual of diagnostic tests for aquatic animals, 6th edition. Office international des épizooties, Paris, p 63-77. Available at: www.oie.int (accessed 1 April 2011)

Pârvulescu L, Paloș C, Molnar P (2009) Firstrecordofthe spiny-cheek crayfish *Orconectes limosus* (Rafinesque, 1817) (Crustacea: Decapoda: Cambaridae) in Romania. North-West J Zool 5:424−428

Pârvulescu L, Petrescu I (2010) The distribution of stone crayfish Austropotamobius torrentium (Schrank, 1803) (Crustacea: Decapoda: Astacidae) in the south-west Romanian mountain and sub-mountain area. Trav Mus Natl Hist Nat 'Grigore Antipa' 53:103−113

Peay S (2009a) Selection criteria for 'ark sites' for white-clawed crayfish. In: Brickland J, Holdich DM, Imhoff EM (eds) Crayfish conservation in the British Isles. Proceedings of conference held in Leeds, p 63−69. Available at: http://iz.carnegiemnh.org/crayfish/IAA/docs/2009_Crayfish_Conservation_in_the_British_Isles_LR.pdf

Peay S (2009b) Invasive non-indigenous crayfish species in Europe: recommendations on managing them. Knowl Manag Aquat Ecosyst 394−395:9

Persson M, Söderhäll K (1983) *Pacifastacus leniusculus* Dana and its resistance to the parasitic fungus *Aphanomyces astaci* Schikora. Freshw Crayfish 5:292−298

Petrusek A, Filipová L, Ďuriš Z and others (2006) Distribution of the invasive spiny-cheek crayfish (*Orconectes limosus*) in the Czech Republic. Past and present. Bull Fr Peche Piscicult 380−381:903−917

Puky M (2009) Confirmation of the presence of the spiny-cheek crayfish *Orconectes limosus* (Rafinesque, 1817) (Crustacea: Decapoda: Cambaridae) in Slovakia. North-West J Zool 5:214−217

Puky M, Schád P (2006) *Orconectes limosus* colonises new areas fast along the Danube in Hungary. Bull Fr Peche Piscicult 380−381:919−926

Schulz HK, Smietana P, Maiwald T, Oidtmann B, Schulz R (2006) Case studies on the co-occurrence of *Astacus astacus* (L.) and *Orconectes limosus* (Raf.): snapshots of a slow displacement. Freshw Crayfish 15:212−219

Skov C, Aarestrup K, Sivebæk F, Pedersen S, Vrålstad T, Berg S (2011) Non-indigenous signal crayfish (*Pacifastacus leniusculus*) are now common in Danish streams: preliminary status for national distribution and protective actions. Biol Invasions 13:1269−1274

Skurdal J, Taugbøl T (2002) *Astacus*. In: Holdich DM (ed) Biology of freshwater crayfish. Blackwell Science, Oxford, p 467−510

Söderhäll K, Cerenius L (1999) The crayfish plague fungus: history and recent advances. Freshw Crayfish 12:11−35 Strand DA, Holst-Jensen A, Viljugrein H, Edvardsen B, Klaveness D,Jussila J, Vrålstad T (2011) Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. Dis Aquat Org 95:9−17

Svoboda J, Kozubíková E, Kozák P, Kouba A and others (2012) PCR detection of the crayfish plague pathogen in narrow-clawed crayfish inhabiting Lake Eg ̆ırdir in Turkey. Dis Aquat Org (in press)

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731−2739

Taugbøl T, Skurdal J, Håstein T (1993) Crayfish plague and management strategies in Norway. Biol Conserv 63: 75−82

Tuffs S, Oidtmann B (2011) A comparative study of molecular diagnostic methods designed to detect the crayfish plague pathogen, *Aphanomyces astaci*. Vet Microbiol 153:343−353

Unestam T (1969) Resistance to the crayfish plague in some American, Japanese and European crayfishes. Rep Inst Freshw Res Drottningholm 49:202−209

Vey A, Söderhall K, Ajaxon R (1983) Susceptibility of *Orconectes limosus* Raff to the crayfish plague, *Aphanomyces astaci* Schikora. Freshw Crayfish 2:284−291

Vrålstad T, Knutsen AK, Tengs T, Holst-Jensen A (2009) A quantitative TaqMan (R) MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. Vet Microbiol 137:146−155

Vrålstad T, Johnsen SI, Fristad RF, Edsman L, Strand D (2011) Potent infection reservoir of crayfish plague now permanently established in Norway. Dis Aquat Org 97:75−83

Westman K, Savolainen R, Julkunen M (2002) Replacement of the native crayfish *Astacus astacus* by the introduced species *Pacifastacus leniusculus* in a small, enclosed Finnish lake: a 30-year study. Ecography 25:53−73

Aquatic Invasions (2012) **7**(4): 503–510.

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

**Anne Schrimpf[1], Lucian Pârvulescu[2], Denis Copilaș-Ciocianu[2], Adam Petrusek[3] and Ralf Schulz[1]**

[1] *University Koblenz-Landau, Institute for Environmental Sciences, 76829 Landau, Germany*
[2] *West University of Timisoara, Faculty of Chemistry, Biology, Geography, Department of Biology and Chemistry, 300115, Timisoara, Romania*
[3] *Charles University in Prague, Faculty of Science, Department of Ecology, 12844 Prague, Czech Republic*

## 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.

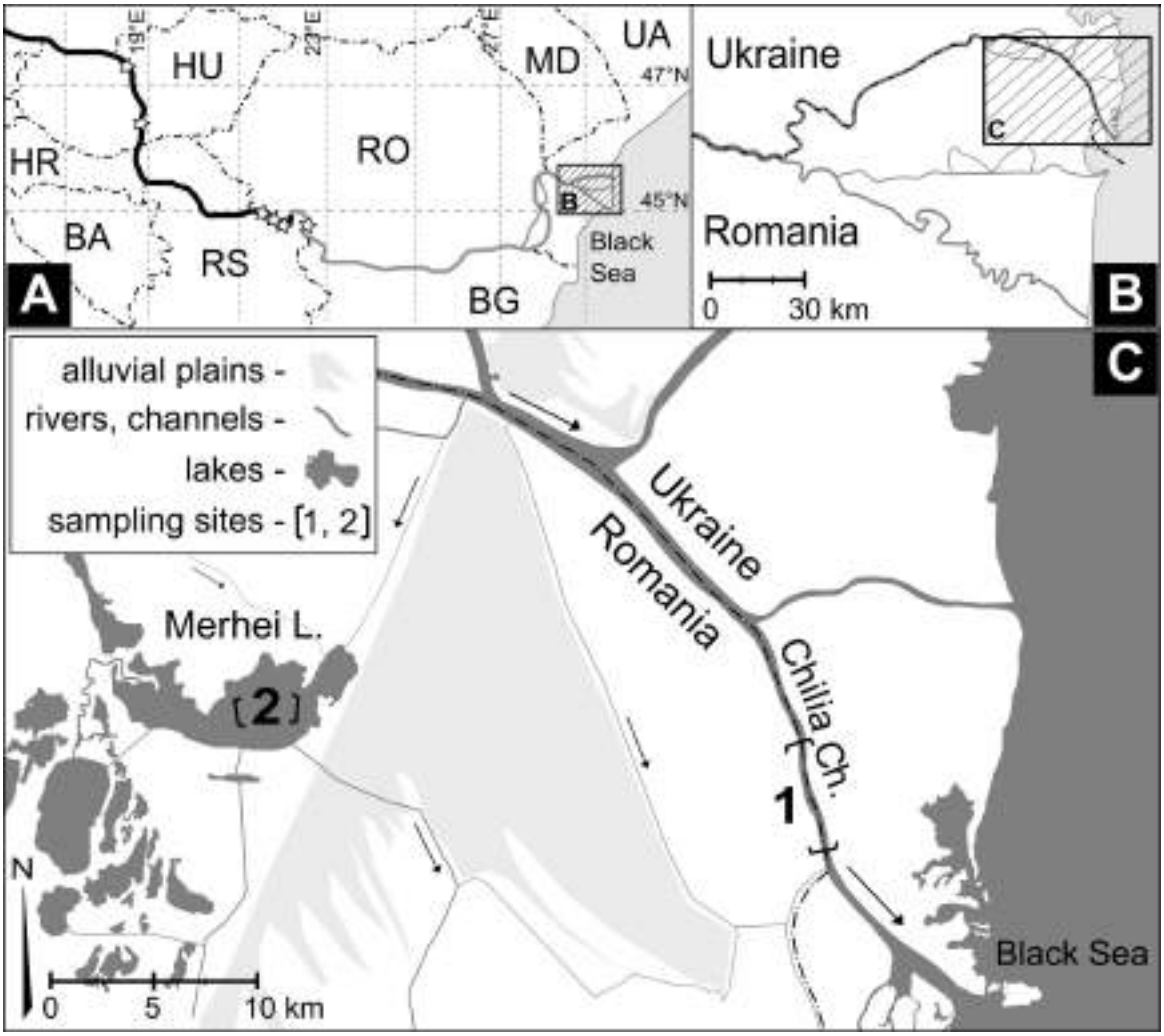


**Figure 1. A**: Overview of the distribution of *Orconectes limosus* (dark line) and the sites with confirmed presence of the crayfish plague pathogen (stars) in the Danube; **B**: Overview of the Danube Delta; **C**: Sampling sites in the Danube Delta (1- Chilia Channel, 2- Merhei Lake). The arrows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

## 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 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- 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.

**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*.

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.

## ACKNOWLEDGEMENTS

The field investigations and equipment were financially supported by the Bio-Team Association (Timisoara, Romania). Many thanks are due to Administration of the Danube Delta Biosphere Reservation for the permission to conduct this study. We thank Nora Veidt and Doreen Roblick for help in the laboratory, and two anonymous referees for comments on the manuscript. Publication of this paper was supported by the European Commission 7th Framework Programme through the *enviroGRIDS* project (Grant Agreement No. 26740).

## REFERENCES


Alderman DJ, Polglase JL, Frayling M (1987) *Aphanomyces astaci* pathogenicity under laboratory and field conditions. *Journal of Fish Diseases* 10: 385-393, http://dx.doi.org/10.1111/j.1365-2761.1987.tb01086.x

Alderman DJ (1996) Geographical spread of bacterial and fungal diseases of crustaceans. *Revue Scientifique et Technique - Office International des Epizooties* 15: 603-632

Harlioğlu MM (2008) The harvest of the freshwater crayfish *Astacus leptodactylus* Eschscholtz in Turkey: harvest history, impact of crayfish plague, and present distribution of harvested populations. *Aquaculture International* 16: 351-360, http://dx.doi.org/10.1007/s10499-007-9145-7

Hewitt G (1999) Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society* 68: 87-112, http://dx.doi.org/10.1111/j.1095-8312.1999.tb01160.x

Holdich DM (2002) Distribution of crayfish in Europe and some adjoining countries. *Bulletin Français de la Pêche et de la Pisciculture* 367: 611-650, http://dx.doi.org/10.1051/kmae:2002055

Holdich DM, Haffner P, Noël PY (2006) Species Files. In: Souty-Grosset C, Holdich DM, Reynolds JM, Haffner P (eds), Atlas of Crayfish in Europe. Publications Scientifiques, Paris, France, pp 66-71

Holdich DM, Reynolds JD, Souty-Grosset C, Sibley PJ (2009) A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowledge and Management of Aquatic Ecosystems* 394-395: 11, http://dx.doi.org/10.1051/kmae/2009025

Huang TS, Cerenius L, Söderhäll K (1994) Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci,* by random amplification of polymorphic DNA. *Aquaculture* 126: 1-9, http://dx.doi.org/10.1016/0044-8486(94)90243-7

Hudina S, Faller M, Lucić A, Klobučar G, Maguire I (2009) Distribution and dispersal of two invasive crayfish species in the Drava River basin, Croatia. *Knowledge and Management of Aquatic Ecosystems* 394-395: 09

Jażdżewski K, Konopacka A, Grabowski M (2005) Native and alien malacostracan Crustacea along the Polish Baltic Sea coast in the 20th century. *Oceanological and Hydrobio-logical Studies* 34: 175-193

Jussila J, Makkonen J, Vainikka A, Kortet R, Kokko H (2011) Latent crayfish plague (*Aphanomyces astaci*) infection in a robust wild noble crayfish (*Astacus astacus*) population. *Aquaculture* 321: 17-20, http://dx.doi.org/10.1016/j.aqua culture.2011.08.026

Keller RP, Frang K, Lodge DM (2008) Preventing the spread of invasive species: economic benefits of intervention guided by ecological predictions. *Conservation Biology* 22: 80-88, http://dx.doi.org/10.1111/j.1523-1739.2007.00811.x

Kozák P, Füreder L, Kouba A, Reynolds JD, Souty-Grosset C (2011) Current conservation strategies for European crayfish. *Knowledge and Management of Aquatic Ecosystems* 401: 01

Kokko H, Koistinen L, Harlioğlu MM, Makkonen J, Aydın H, Jussila J (2012) Recovering Turkish narrow clawed crayfish (*Astacus leptodactylus*) populations carry *Aphanomyces astaci*. Knowledge and Management of Aquatic Ecosystems 404: 12.

Kozubíková E, Filipová L, Kozák P, Ďuriš Z, Martín MP, Diéguez-Uribeondo J, Oidtmann B, Petrusek A (2009) Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in invasive American crayfishes in the Czech Republic. *Conservation Biology* 23: 1204-1213,

Kozubíková E, Puky M, Kiszely P, Petrusek A (2010) Crayfish plague pathogen in invasive North American crayfish species in Hungary. *Journal of Fish Diseases* 33: 925-929, http://dx.doi.org/10.1111/j.1365-2761.2010.01199.x

Kozubíková E, Viljamaa-Dirks S, Heinikainen S, Petrusek A (2011a) Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague agent *Aphanomyces astaci. Journal of Invertebrate Pathology* 108: 214-216, http://dx.doi.org/10.1016/j.jip.2011.08.002

Kozubíková E, Vrålstad T, Filipová L, Petrusek A (2011b) Re-examination of the prevalence of *Aphanomyces astaci* in North American crayfish populations in Central Europe by TaqMan MGB real-time PCR. *Diseases of Aquatic Organisms* 97: 113-125, http://dx.doi.org/10.3354/dao02411

Lowe S, Browne M, Boudjelas S, De Poorter M (2004) 100 of the world's worst invasive alien species. A selection from the Global Invasive Species Database. The Invasive Species Specialist Group (ISSG), a specialist group of the Species Survival Commission (SSC) of the IUCN, Gland, Switzerland and reprinted version: November 2004

Nylund V, Westman K (2000) The prevalence of crayfish plague (*Aphanomyces astaci*) in two signal crayfish (*Pacifastacus leniusculus*) populations in Finland. *Journal of Crustacean Biology* 20: 777-785, http://dx.doi.org/10.1651/0278-0372 (2000)020[0777:TPOCPA]2.0.CO;2

Nyström P (1999) The effects of crayfish on interactions in freshwater benthic communities. PhD thesis, Lund University, Lund, Sweden

Oidtmann B, Heitz E, Rogers D, Hoffmann RW (2002) Transmission of crayfish plague. *Diseases of Aquatic Organisms* 52: 159-167, http://dx.doi.org/10.3354/dao052159

Pârvulescu L, Paloș C, Molnar P (2009) First record of the spiny-cheek crayfish *Orconectes limosus* (Rafinesque, 1817) (Crustacea: Decapoda: Cambaridae) in Romania. *North-Western Journal of Zoology* 5: 424-428

Pârvulescu L, Schrimpf A, Kozubíková E, Resino SC, Vrålstad T, Petrusek A, Schulz R (2012) Invasive crayfish and crayfish plague on the move: first detection of the plague agent *Aphanomyces astaci* in the Romanian Danube. *Diseases of Aquatic Organisms* 98: 85-94, http://dx.doi.org/10.3354/dao 02432

Peay S (2009) Selection criteria for "ark sites" for white-clawed crayfish. In: Brickland J, Holdich DM, Imhoff EM (eds) Crayfish Conservation in the British Isles, Proceedings of a conference. March 25th, 2009. British Waterways Offices, Leeds, UK, pp 63-69

Puky M, Schád P (2006) *Orconectes limosus* colonises new areas fast along the Danube in Hungary. *Bulletin Français de la Pêche et de la Pisciculture* 380-381: 919-926.

Schulz HK, Smietána P, Maiwald T, Oidtmann B, Schulz R (2006) Case studies on the co-occurrence of *Astacus astacus* (L.) and *Orconectes limosus* (Raf.): snapshots of a slow displacement. *Freshwater Crayfish* 15: 212-219

Schrimpf A, Schulz HK, Theissinger K, Pârvulescu L, Schulz R (2011) The first large-scale genetic analysis of the vulnerable noble crayfish *Astacus astacus* reveals low haplotype diversity in central European populations. *Knowledge and Management of Aquatic Ecosystems* 401: 35

Strand DA, Holst-Jensen A, Viljugrein H, Edvardsen B, Klaveness D, Jussila J, Vrålstad T (2011) Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. *Diseases of Aquatic Organisms* 95: 9-17, http://dx.doi.org/10. 3354/dao02334

Svoboda J, Kozubíková E, Kozák P, Kouba A, Bahadir Koca S, Diler Ö, Diler I, Policar T, Petrusek A (2012) PCR detection of the crayfish plague pathogen in narrow-clawed crayfish inhabiting Lake Eğirdir in Turkey. *Diseases of Aquatic Organisms* 98: 255-259, http://dx.doi.org/10.3354/dao02445

Trontelj P, Machino Y, Sket B (2005) Phylogenetic and phylogeographic relationships in the crayfish genus *Austropotamobius* inferred from mitochondrial COI gene sequences. *Molecular Phylogenetics and Evolution* 34: 212-226, http://dx.doi.org/10.1016/j.ympev.2004.09.010

Tuffs S, Oidtmann B (2011) A comparative study of molecular diagnostic methods designed to detect the crayfish plague pathogen, *Aphanomyces astaci. Veterinary Microbiology* 153: 343-353, http://dx.doi.org/10.1016/j.vetmic.2011.06.012

UNESCO World Heritage Centre (2012) Danube Delta. http://whc.unesco.org/en/list/588/ (Accessed 31 January 2012)

Unestam T (1969) On the adaptation of *Aphanomyces astaci* as a parasite. *Physiologia Plantarum* 22: 221-235

Viljamaa-Dirks S, Heinikainen S, Nieminen M, Vennerström P, Pelkonen S (2011) Persistent infection by crayfish plague *Aphanomyces astaci* in a noble crayfish population – a case report. *Bulletin of the European Association of Fish Pathologists* 31: 182-188

Vrålstad T, Knutsen AK, Tengs T, Holst-Jensen A (2009) A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci. Veterinary Microbiology* 137: 146-155, http://dx.doi.org/10.1016/j.vet mic.2008.12.022

Vrålstad T, Johnsen S, Fristad R, Edsman L, Strand D (2011) Potent infection reservoir of crayfish plague now permanently established in Norway. *Diseases of Aquatic Organisms* 97: 75-83, http://dx.doi.org/10.3354/dao02386

Freshwater Biology (in press)

# *Absence of the crayfish plague pathogen (Aphanomyces astaci) facilitates coexistence of European and American crayfish in central Europe*

Running head: Plague absence support crayfish coexistence

**Anne Schrimpf[1], Torsten Maiwald[1], Trude Vrålstad[2,3], Holger K. Schulz[1], Przemysław Smietána[4] and Ralf Schulz[1]**

[1] University Koblenz-Landau, Institute for Environmental Sciences, Fortstrasse 7, 76829 Landau, Germany
[2] Norwegian Veterinary Institute, Pb 750, Sentrum, 0106 Oslo, Norway
[3] University of Oslo, Department of Biology, Microbial Evolution Research Group, Pb 1066, Blindern, 0316 Oslo, Norway
[4] Uniwersytet Szczeciński, ul. Wąska 13, 71-415 Szczecin, Polen

## SUMMARY

Most European crayfish species are strongly threatened, mainly as a result of the introduced crayfish plague agent, *Aphanomyces astaci*, transmitted by invasive North American crayfish. Long-term coexistence of American and European crayfish species is therefore regarded as almost impossible, even thought some coexisting populations have been observed.

In this study, crayfish were collected from presently coexisting populations of the introduced spiny-cheek crayfish (*Orconectes limosus*) and the native noble crayfish (*Astacus astacus*) from nine standing waters in central Europe. Our aim was to resolve whether the coexistence resulted from reduced virulence in local strains of *A. astaci*, increased immunity in the native crayfish, or an absence of the pathogen in these populations. We used highly sensitive *A. astaci*-specific real-time PCR to evaluate the crayfish latent carrier status, combined with transmission experiments to further validate the molecular results.

From the total of 523 crayfish tested (490 spiny-cheek crayfish, 33 noble crayfish), none positive for *A. astaci* were detected. Transmission experiments confirmed these results: No abnormal mortality or behavioural changes were seen in noble crayfish kept together with American crayfish from the coexisting populations. If we assume a very low prevalence of *A. astaci* of 10% in a carrier population, there is a 98% probability of disease absence in five of the nine

coexisting populations tested. Hence, a consistent absence, or an extremely low prevalence of *A. astaci*, seems to allow the coexistence of European and American crayfish in these central European populations.

The results are important for native crayfish conservation and management, and demonstrate that disease transmission risk may vary substantially between the different populations of spiny-cheek crayfish in central Europe.

**Keywords:** Crayfish plague • Invasive species • Coexistence • Real-time PCR • transmission experiment

## INTRODUCTION

Land use, climate and biotic exchange are the main drivers of the loss of freshwater biodiversity (Sala *et al*., 2000). The introduction of alien species is thought to have a higher impact on biodiversity in aquatic than in terrestrial ecosystems, and particularly in lakes (Sala *et al*., 2000). Crayfish are the largest, mobile freshwater invertebrates, occupying lakes, rivers and streams. They are considered keystone species with a high biological impact on their environment due to their omnivory (Holdich, 2002; Reynolds & Souty-Grosset 2012*).* Today, the existence of all the native European crayfish species is gravely threatened by the introduction and spread of alien species. The crayfish plague pathogen, *Aphanomyces astaci* (Oomycetes), carried and transmitted by North-American crayfish species is the major cause of the sharp decline in native crayfish populations in Europe*.* While this obligate crayfish parasite lives in a balanced host-parasite relationship with North American crayfish, it is a lethal pathogen of native European crayfish, usually leading to 100% mortality (OIE, 2012). Therefore, this aggressive pathogen is listed among the world´s 100 worst alien species (Lowe *et al*., 2004). *Aphanomyces astaci* originates from North America (Unestam, 1972), but has repeatedly been brought into Europe along with introduction of American crayfish species (Alderman, 1996).

If American crayfish species are stressed or immunocompromised as a result of moulting, infection by other pathogens or an adverse environment, they too may succumb to crayfish plague (Persson *et al*., 1987; Söderhäll & Cerenius,

1992). However, they normally cope with *A. astaci* due to evolved defence mechanisms involving melanin encapsulation of the parasite that prevents further spread within the crayfish (Unestam, 1969; Söderhall & Cerenius, 1999). Consequently, the animals can be clinically healthy, but still act as infection reservoirs of *A. astaci* (Söderhall & Cerenius, 1999; Taugbol, 2004; Holdich *et al*., 2009; OIE, 2009). Four species of North American crayfish introduced to Europe are confirmed carriers of the disease: i.e. the spiny-cheek crayfish (*Orconectes limosus*), the signal crayfish (*Pacifastacus leniusculus*), the red swamp crayfish (*Procambarus clarkii*) (Holdich *et al*., 2009), and the calico crayfish (*Orconectes immunis*) (Schrimpf *et al*., 2013). *Orconectes limosus* was the first known carrier of *A. astaci* in Europe (Kozubiková *et al*., 2011a). It was introduced to Poland and Germany around 1890, and is now wide spread in 21 countries (Holdich *et al.*, 2009).

All North American crayfish should according to the OIE (2009) be regarded as likely carriers of *A. astaci*. This, together with the catastrophic decline of native European crayfish (Bohman, Nordwall & Edsman, 2006, Holdich *et al*., 2009), suggests that the stable coexistence of American and European crayfish species is unlikely. However, examples of apparently long-term coexistence have been found ocassionally for signal crayfish and noble crayfish (*Astacus astacus*) in Finland (Westman & Savolainen, 2001; Westman, Savolainen & Julkunen, 2002) and Sweden (Söderbäck 1994), for spiny-cheek crayfish and native narrow-clawed crayfish (*Astacus leptodactylus*) in Romania (Pârvulescu *et al*., 2012), and for spiny-cheek crayfish and noble crayfish in lakes from Poland (Schulz *et al*., 2006). Indications of pathogen free American crayfish populations have also been found sporadically (Matasová *et al*., 2011; Skov *et al*., 2011). Schulz *et al*. (2006) suggest that the displacement of noble crayfish by spiny-cheek crayfish in some lakes in Poland is caused by direct competition instead of via *A. astaci* infection of the European species. A replacement of noble crayfish was also proposed by Westman *et al*. (2002) for a long-term coexisting population of signal crayfish and noble crayfish in Finland.

The coexistence of American and European crayfish species might be possible if some European crayfish have evolved resistance against the pathogen.

In this case, the pathogen should be found in the population. The same would apply if a less virulent strain of *A. astaci* had evolved that is not lethal to European crayfish species. It has been shown that different North American crayfish species are carriers of different genotypes of *A. astaci* (Huang, Cerenius & Söderhäll, 1994; Diéguez-Uribeondo, Cerenius & Söderhäll, 1995, Kozubíková *et al*., 2011a) and that these genotypes may vary in their virulence (Viljamaa-Dirks *et al*., 2011). Makkonen *et al*. (2012) assume that the high genetic diversity of *A. astaci* chitinase genes may indicate its adaption to specific host species.

These new results may explain why a few populations of native European crayfish are resistant to some extent against the pathogen, as has been demonstrated recently in a population of noble crayfish in Finland where the *A. astaci* genotype group A, or As-type, was isolated in pure culture (Viljamaa-Dirks *et al*., 2011). The As-genotype has only been isolated from noble crayfish and hence the original American host species in unknown (Huang *et al.*, 1994). The high polymorphism of the As-genotype, and coevolution with noble crayfish may explain its adaptation to native European crayfish species (Makkonen *et al*., 2012). Other recent papers on the basis of molecular evidence suggest that persistent infections can occur in native crayfish populations (Jussila *et al*., 2011; Viljamaa-Dirks *et al*., 2011; Kokko *et al.*, 2012; Schrimpf *et al*., 2012; Svoboda *et al*., 2012). A third scenario that may allow the European species to coexist with American species could be the complete absence of the pathogen in these stocks. In that case, all tests for the presence of the pathogen should be negative.

In the present study, we aimed to test which of these three hypotheses (i.e. a) resistant native crayfish, b) reduced pathogenicity, c) no pathogen) may apply to the apparently long-term coexistence of the North American spiny-cheek crayfish and the native noble crayfish in several populations in central Europe. We used quantitative real-time PCR to clarify the *A. astaci* carrier status in the coexisting stocks of nine waters in Germany and Poland, and strengthened this molecular analysis with classic transmission experiments.

## METHODS

*Study Sites and Crayfish Sampling*

With the help of local fishery administrations in Germany and Poland, coexisting populations of spiny-cheek crayfish and noble crayfish were localized. Between 2006 and 2011, we trapped noble crayfish and spiny-cheek crayfish, between July and September each year from five and four lakes in Poland and in Germany, respectively, where the species coexist (Table 1). The two species have coexisted in these lakes at least since 2006, when this study was started, and no exceptional mortalities of noble crayfish (indicating a plague outbreak) have been reported since then. In the five Polish lakes, the first records of the coexistence are from 2000 (Milaczewo), 2001 (Piasek, Trzcinskie), 2003 (Zielone) and 2006 (Plociowe). When possible we collected the invasive crayfish species without harm the native noble crayfish. The lakes are between approximately 5 and 50 ha surface area. We also collected spiny-cheek crayfish from two German and Polish lakes (Plociowe and Hennweiler), where no noble crayfish have been observed for at least one decade. We also collected signal crayfish from the Heimbach, a German river where native species are no longer present. These individuals where primarily included as positive controls for demonstrating the expected positive carrier status for American crayfish. Finally, we took noble crayfish from the “First Bavarian Crayfish hatchery” in Augsburg, Germany as a negative control (Table 2). This hatchery has been surveyed for mortalities of noble crayfish very carefully before and after sampling, and none has been reported for more than 20 years (Max Keller, pers. comm).

*Molecular analyses*

Reliable evaluation of the presence or absence of the crayfish plague pathogen is best achieved with molecular diagnostics. A molecular screening of American and European crayfish using the agent specific and quantitative TaqMan® minor groove binder (MGB) real-time PCR (qPCR; Vrålstad *et al*., 2009) was performed in order to detect putative *A. astaci* infections. The assay has demonstrated very high sensitivity and specificity for *A. astaci*, and is recommended for evaluating the carrier status of American crayfish (Tuffs &

Oidtmann, 2011). A total of 490 spiny-cheek crayfish and 33 noble crayfish were tested from coexisting populations, and eight noble crayfish were tested from the crayfish farm in Augsburg (Tables 1 & 2). As indicated above, spiny-cheek crayfish and signal crayfish from the single species lakes (Table 2), were included in the qPCR analyses as positive controls.

Due to two different time periods of processing and qPCR analyses of the data included in this study, we will in the following refer to two sample sets that were processed separately (A and B, Tables 1 & 2). For sample set A (244 samples), only the soft abdominal cuticle was used for DNA-extraction. Here, the cuticle was cut into two halves longitudinally and the two samples tested. For sample set B (319 samples), three tissue types were included (abdominal cuticle, uropods and the inner joint of a walking leg according to Vrålstad *et al*., 2011). After each dissection, tools were disinfected using DNAexitus (AppliChem GmbH, Germany). The DNA was extracted according to Vrålstad *et al*. (2009). An extraction blank control not including tissue material was included in each DNA extraction setup, and an environmental control consisting of 200 µl nuclease free water that was left open during the extraction procedure. Both types of control were included in the further qPCR analyses. The qPCR procedure was performed according to Vrålstad *et al*. (2009) with some modifications. The qPCR for sample set A was run on a MJ research Chromo4, and the Universal PCR Master Mix was replaced by 1 u GoTaq DNA Polymerase (Promega, Germany), 1× Colourless Go Taq® Flexi Buffer (Promega, Germany), 2.5 mM $MgCl_2$ (Promega, Germany), 0.2 mM of each dNTP (Fermentas, Germany) and 2 µg BSA (Fermentas, St. Leon-Rot, Germany). For this sample set, agent levels were assigned on the basis of corresponding Ct-values (Vrålstad *et al*., 2009; Kozubíková *et al*., 2011b). Sample set B was run on a Mx3005P qPCR system (Stratagene, USA) using the TaqMan® Environmental Master Mix (Applied Biosystems, USA) to avoid PCR inhibition (Strand *et al*., 2011). For half of the sample set B, the annealing temperature was increased to 62°C and the annealing time decreased to 15 seconds to further increase the assay specificity (T. Vrålstad, unpubl. data). Data analysis was carried out using MxPro software ver. 4.10 (Stratagene, USA) and Opticon Monitor 2.03 (MJ Research, Canada). DNA-

extracts were tested in duplicates as concentrated (1x) and 5x diluted templates. A no-template PCR control consisting of 5 µl nuclease free water was included in each run together with a standard series of genomic DNA from a pure *A. astaci* culture. The standard series (a four-fold dilution series) included four calibrant points for each run, and was used to assign positive results to semi-quantitative agent levels according to Vrålstad *et al*. (2009).

*Statistics*

A statistical test was conducted in R v.2.12.2 (R Development Core Team, 2011) using the function epi.detectsize in the package epiR (Stevenson *et al*., 2010) to estimate the probability of the absence of disease (i.e. *A. astaci*). The function computes the number of random samples (individuals) required to declare a population free from the crayfish plague agent at a certain confidence level depending on the expected prevalence of the pathogen in an infected population (or the theoretical prevalence level we want to be able to reveal), the given population size (N), test sensitivity (Se) and specificity (Sp) (Dohoo, Martin & Stryhn, 2003).

If we assume a mean population density of two individuals $m^{-2}$ for an unexploited population (Abrahamsson, 1966), the population size (including crayfish of all size classes) varies between $10^5$ individuals for a 5 ha lake and $10^6$ individuals for a 50 ha lake. We do not know if the population sizes for mixed coexisting populations vary, therefore the test was run for different hypothetical population size estimates that should apply to coexisting populations as well as to single species lakes ($N = 10^3$; $N = 10^4$; $N = 10^5$; $N = 10^6$). Expected prevalence (Prev = % positive individuals in a population), including the smallest prevalence we want to be able to reveal, was also tested with different estimates (high Prev = 0.8; low Prev = 0.3; very low Prev = 0.1; exceptionally low Prev = 0.01). The real-time method used has very high sensitivity (Vrålstad *et al*., 2009; Tuffs & Oidtmann, 2011). While analysis of the uropod tissue alone detected 84% of the total of 86% positives of signal crayfish, analyses of the soft abdominal cuticle targeted only 30% (Vrålstad *et al*., 2011). Notably, in spiny-cheek crayfish, the soft abdominal cuticle was most frequently positive (85% positives of a total of

92% infected specimen; Oidtman *et al*., 2006). Therefore, a test sensitivity of 0.8 seems realistic for spiny-cheek crayfish, even when only the cuticle is analysed. However, to account for various sensitivity scenarios, we included three different sensitivity estimates (0.3; 0.5; 0.8) in the statistical tests. Specificity was set to 0.99, since the real-time method used has proved to be very specific and detects all known genotypes of *A. astaci* (Vrålstad *et al*., 2009; Tuffs & Oidtmann, 2011). The test was run for two confidence levels (0.98 and 0.95).

*Transmission experiments*

In the transmission experiment noble European crayfish susceptible to *A. astaci* and potentially infected American crayfish were kept together. High mortality and behavioural change of the native species indicates a positive carrier status of the American crayfish. Spiny-cheek crayfish from German and Polish stocks were kept under controlled conditions, together with noble crayfish from the crayfish farm in Augsburg (Germany) (Table 3) for 63 and 91 days, respectively. From each of the coexisting populations, and from the single species population from Plociowe, we placed six crayfish (three spiny-cheek crayfish and three noble crayfish from the crayfish farm, the latter one known not to be infected) in a separate aquarium with a bottom area of 0.125 $m^2$ (31.25 L) to form a coexisting crayfish assemblage with a population density of 48 individuals $m^{-2}$. As soon as an animal died, it was replaced to keep the population density stable. Dead animals were frozen and kept for subsequent verification of *A. astaci* by molecular diagnostics. Fine gravel was used as substratum in the aquarium. No crayfish shelter was offered in order to increase stress and thereby weaken of the immune system. We used regular tap water with the following mean measured water quality values: Sodium = 3.2 mg $L^{-1}$, potassium = 2.1 mg $L^{-1}$, calcium = 27.5 mg $L^{-1}$, magnesium = 6.9 mg $L^{-1}$, aluminium = 0.020 mg $L^{-1}$, iron = 0.030 mg $L^{-1}$, ammonium = 0.07 mg $L^{-1}$, nitrate = 2.0 mg $L^{-1}$, nitrite ≤ 0.05 mg $L^{-1}$, chloride = 6.3 mg $L^{-1}$, sulfate = 15.8 mg $L^{-1}$, total organic carbon < 1 mg $L^{-1}$. Water temperature (18 – 25°C), oxygen content (7.0 – 9.6 mg $L^{-1}$) and pH (7.7 – 8.4) were monitored over the whole study period and were each in the physiological tolerance range of both crayfish species and the pathogen *A. astaci*.

## RESULTS

*Molecular Diagnostics*

From the total of 523 crayfish (490 spiny-cheek crayfish, 33 noble crayfish) that were tested from the nine localities with coexisting populations, the qPCR diagnostic detected no trace of *A. astaci* in any tissue sample (Table 1). About 56% of the crayfish were tested for three different tissues, while the remaining 44% were tested for the soft abdominal cuticle only. Hence, a total of 1338 crayfish tissue samples were all negative for the coexisting populations. On the contrary, positive status was confirmed in about 66% of the American crayfish tested from single species lakes and a stretch of a river (Table 2). Three out of five spiny-cheek crayfish individuals from Plociowe, where a relatively high noble crayfish mortality was observed in the transmission experiment, were positive (observed prevalence 60%), and all six individuals from Hennweiler were positive (observed prevalence 100%). Furthermore, the pathogen was found in 12 of the 21 signal crayfish tested from Heimbach (observed prevalence 57%). The noble crayfish from the crayfish farm, serving as negative controls, were indeed negative.

**Table 1** Number of crayfish per coexisting population that remained negative for *Aphanomyces astaci* presence by real-time PCR. "-": no data available. The geographical coordinates of the study sites can be obtained from the corresponding author and have been made available to the chief-editor of *Freshwater Biology.*

| Sampling site | Country | Sample Set | *A. astacus* (number) | *O. limosus* (number) | Total |
|---|---|---|---|---|---|
| Bielener lake* | GER | A | 13 | 91 | 104 |
| Heisteberger Weiher | GER | B | - | 51 | 51 |
| Mastholter lake* | GER | A | 5 | 13 | 18 |
| Milaczcewo* | PO | A | 2 | 34 | 36 |
| Piasek* | PO | 15xA/95xB | 3 | 107 | 110 |
| Steinbruch Roth | GER | B | - | 92 | 92 |
| Lake Stosinko wielkie | PO | B | - | 30 | 30 |
| Trzcinskie* | PO | A | 8 | 16 | 24 |
| Zielone* | PO | 34xA/24xB | 2 | 56 | 58 |
| **Total** | | | **33** | **490** | **523** |

* American crayfish from these coexisting populations were used in transmission experiment with farmed noble crayfish. Tough, the individual from the transmission experiment are not included in this table.

**Table 2** Number of crayfish per reference population tested for *Aphanomyces astaci* presence and according agent level by *A. astaci* specific qPCR. "-": no data available**.**

| Code[a] | Sample Set | Crayfish (number) | Total | Positives (number) | Positives (%) | Agent level[b] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | A0 | A1 | A2 | A3 | A4 |
| | | *A. astacus* | | | | | | | | |
| Cf | A | 8 | 8 | - | - | 8 | 0 | 0 | 0 | 0 |
| | | *O. limosus* | | | | | | | | |
| Pl[c] | A | 5 | 5 | 3 | 60 | 2 | 0 | 1 | 1 | 1 |
| Hw | B | 6 | 6 | 6 | 100 | 0 | 0 | 0 | 4 | 2 |
| | | *P. lenisuculus* | | | | | | | | |
| He | B | 21 | 21 | 12 | 57 | 9 | 0 | 3 | 8 | 1 |

[a] Cf = Crayfish farm (Augsburg), Hw = Hennweiler, He = Heimbach (all Germany), Pl = Plociowe (Poland). Hw, He and Pl = single species lakes.

[b] Agent levels refer to semi-quantitative categories based on the numbers of observed PCR forming units ($PFU_{obs}$) from the *A. astaci*−specific real-time PCR. Agent Level 0 (A0): negative; (A1): below the limit of detection ($PFU_{obs} < 5$); A2: $5 \leq PFU_{obs} < 50$; A3: $50 \leq PFU_{obs} < 103$; A4: $103 \leq PFU_{obs} < 104$ (according to Vrålstad *et al*., 2009).

[c] Spiny-cheek crayfish from the Plociowe site used in transmission experiment with farmed noble crayfish.

*Transmission Experiment*

The molecular results were supported by the transmission experiment. A behavioural change (lack of coordination, loss of escape reflex) in noble crayfish indicating crayfish plague infection was observed in the aquarium stocked with spiny-cheek crayfish from the single species lake Plociowe (Table 3). The proportion of dead noble crayfish was relatively high, with 0.07 dead individuals $d^{-1}$. In the six aquaria housing spiny-cheek crayfish that originated from the six coexisting populations the noble crayfish mortality was lower (in three aquaria 0.02 $d^{-1}$ and in the other three aquaria 0.03 $d^{-1}$) (Table 3). The qPCR analysis demonstrated that noble crayfish kept in an aquarium with spiny-cheek crayfish from the sampling site Plociowe were infected by *A. astaci*, while similar tests remained negative for the other noble crayfish that had been exposed to spiny-cheek crayfish from the co-existing populations.

**Table 3** Number of dead *Astacus astacus* during the experiment duration (63 to 91 days) in transmission experiments with *Orconectes limosus* from the studied sampling sites in Germany (GER) and Poland (PO).

| Sampling site | Country | Coexistence | Dead *A. astacus* (Individuals/day) | Observed behavioral change |
|---|---|---|---|---|
| Bielener lake | GER | + | 0.02 | - |
| Mastholter lake | GER | + | 0.03 | - |
| Milaczewo | PO | + | 0.02 | - |
| Piasek | PO | + | 0.03 | - |
| Trzcinskie | PO | + | 0.03 | - |
| Zielone | PO | + | 0.02 | - |
| Plociowe | PO | - | 0.07 | + |

*Probability of Absence of the Pathogen*

Calculations of the required number of crayfish individuals analysed to estimate the probability of absence of *A. astaci* are listed in Table 4. The number strongly depends on the assumed prevalence and test sensitivity, while population size affects these numbers to a lower extent, at least if N is high. With the highest tested estimates for prevalence (80% positives), sensitivity (0.8), and population size ($N = 10^6$), positives will be detected if four individuals are tested (98% confidence level). With the lowest tested estimates for prevalence (1% positives) and test sensitivity (0.3), but the highest population size ($N = 10^6$), ~1300 individuals must remain negative before the pathogen can be declared absent with 98% confidence. The numbers tested in the current study vary with locations. If we assume 80% test sensitivity, comparable to results in Vrålstad *et al*. (2011), and 10% as the lowest probable prevalence of *A. astaci* positive individuals in an American crayfish population, five of the nine coexisting spiny-cheek crayfish and noble crayfish populations tested in this study (i.e.≥ 47 tested crayfish) (Table 1) can be declared pathogen free with 98% confidence (Table 4). Further, if *A. astaci* was present, but undetected, in any of the tested coexisting populations (Table 1), the prevalence would in all cases be less than 30% (i.e.≥ 14 tested crayfish required to detect one positive at 98% confidence level; Table 4).

**Table 4** The required sample sizes, calculated from the epi.detectsize function in the epiR package in R, to confirm absence of disease/negative carrier status at a given confidence level (98% and 95%) given all samples are negative.

| Test parameters[a] | | Samples needed at different population sizes (N) and confidence levels (0.98/0.95) [b] | | | | This study [c] |
|---|---|---|---|---|---|---|
| **Prevalence** | **Sensitivity** | **N = $10^3$** | **N = $10^4$** | **N = $10^5$** | **N = $10^6$** | |
| 0.80 | 0.8 | 4/3 | 4/3 | 4/3 | 4/3 | 9/9 |
| 0.30 | 0.8 | 14/11 | 14/11 | 14/11 | 14/11 | 9/9 |
| 0.1 | 0.8 | 44/34 | 47/36 | 47/36 | 47/36 | 5/5 |
| 0.01 | 0.8 | 278/237 | 453/353 | 484/371 | 487/373 | - |
| 0.80 | 0.5 | 8/6 | 8/6 | 8/6 | 8/6 | 9/9 |
| 0.30 | 0.5 | 23/18 | 24/18 | 24/18 | 24/18 | 7/7 |
| 0.1 | 0.5 | 68/54 | 76/58 | 76/58 | 76/58 | 3/3 |
| 0.01 | 0.5 | 351/310 | 699/548 | 771/592 | 779/597 | - |
| 0.80 | 0.3 | 14/11 | 14/11 | 14/11 | 14/11 | 8/9 |
| 0.30 | 0.3 | 39/30 | 41/32 | 42/32 | 42/32 | 5/6 |
| 0.1 | 0.3 | 107/86 | 126/97 | 128/98 | 128/98 | 0/1 |
| 0.01 | 0.3 | 421/387 | 1088/867 | 1277/982 | 1299/996 | - |

[a] Test parameters in the analysis: prevalence tested from high (0.8), moderate (0.3), low (0.1) to very low (0.01). Sensitivity was tested for high (0.8), medium (0.5) and low (0.3) test sensitivity. Test specificity was set to 99%. Reduced test specificity (down to 50%) did not affect the number of individuals needed.

[b] The test was performed with four different hypothetical population sizes (N = 103, 104, 105 ,106) for two different confidence levels (0.98 and 0.95).

[c] Numbers of coexisting populations out of the nine tested in this study that are demonstrated free of *A. astaci* in spiny-cheek crayfish at given test parameters and confidence levels (0.98 and 0.95). Analysed individuals of noble crayfish are not included in the calculations to avoid assumptions of "equal prevalence" in both species.

## DISCUSSION

### *Pathogen free Coexisting Populations?*

On the basis of tissue analyses with molecular diagnostics (qPCR) combined with traditional transmission experiments we have demonstrated a consistent lack of *A. astaci* positive crayfish individuals in nine coexisting populations of American and European crayfish species in central Europe. To our knowledge, only a few lakes harbouring coexisting American and European crayfish have been recorded (e.g. Westman & Savolainen, 2001; Söderbäck 1994; Schulz *et al*., 2006), and this study is the first to investigate thoroughly the *A. astaci* carrier status in such populations. Many studies have shown that the introduction of American crayfish species is followed by the mass mortality of

susceptible European crayfish species due to transmission of the crayfish plague (e.g. Bohman *et al*., 2006; Kozubíková *et al*., 2008; Vrålstad *et al*., 2011). Some of the key factors that could explain prolonged coexistence could therefore involve either evolved resistance in native crayfish, reduced virulence in the pathogen strain, or absence of the pathogen.

To our knowledge, there has been no demonstration of increased resistance in native European crayfish populations, although recent results do indicate that persistent infections of *A. astaci* in native European crayfish occur (Jussila *et al*., 2011; Viljamaa-Dirks *et al*., 2011; Kokko *et al*., 2012, Svoboda *et al*., 2012; Schrimpf *et al*., 2012). Whether this is due to increased crayfish immunity, or to reduced pathogen virulence, remains to be clarified in most cases. For our study, however, the absence of the pathogen is strongly supported. Only when we assume that a very low prevalence of *A. astaci* in the population is possible (≤5%), combined with low test sensitivity, the sample sizes required to confirm absence of the pathogen increase to unfeasible numbers. Hence, absence, or an extremely low prevalence of the crayfish plague pathogen in these populations stands out as the most likely factor for the observed coexistence.

It is a challenge to test sufficient individuals to exclude the possibility of a very low prevalence of *A. astaci*. As an explanation why some American crayfish populations are *A. astaci*-free it is conceivable that a few pathogen free animals actively immigrated into the coexisting populations, or were passively introduced. Alternatively, environmental or biological changes or factors could have eradicated *A. astaci* from the crayfish populations. Strand *et al*. (2012) recently demonstrated that *A. astaci* qPCR, used directly on water samples from tanks with *A. astaci* positive signal crayfish, is able to detect the pathogen spore content. They found that *A. astaci* spores were continuously released into the water from the carrier crayfish with raised spore levels before and after moulting and mortalities. Hence, screening a large number of individuals could involve analyses of water containing many crayfish. The method detects down to a single spore in the water sample (Strand *et al*., 2011; 2012). It could therefore strengthen our hypothesis of pathogen absence if a large number of individuals from coexisting

populations were kept in a common tank over time without releasing a detectable number of *A. astaci* spores into the water.

*Pathogen Prevalence*

Before results from molecular screenings of American crayfish populations became available, it was generally assumed and expected that all American crayfish are carriers of *A. astaci* (Unestam, 1972; Oidtmann *et al*., 2006, OIE, 2009). Many more recent studies have investigated the carrier status of American crayfish. However, the crayfish number and tissue types tested vary greatly, making comparisons difficult. Highly variable prevalence has been reported using the Vrålstad *et al*. (2009) qPCR method, from 0-100% in *O. limosus* and 0-37% in *P. leniusculus* (Kozubíková *et al*., 2011b), but here only soft abdominal cuticle was analysed in a variable number of individuals per population.

Three comparable studies testing 20-40 crayfish individuals per population and three tissues per crayfish (cuticle, uropods and walking legs) also demonstrated extremely variable prevalence. In one of the studies, no positive individuals were found in populations of signal crayfish and noble crayfish coexisting in the same river in Denmark (Skov *et al*., 2011), while in the other studies, the observed prevalence of *A. astaci* in a signal crayfish populations were 86%, 90% and 100% for one Norwegian and two Finnish populations, respectively (Vrålstad *et al*., 2011; Strand *et al*., 2012). In the Norwegian case, the high prevalence explained the recent crayfish plague outbreaks in native noble crayfish populations in this watercourse (Vrålstad *et al*., 2011). In our study, the prevalence of spiny-cheek crayfish positive for *A. astaci* in the populations from Plociowe and Hennweiler was 60% and 100%, respectively. The lower prevalence limit for an obligate crayfish parasite like *A. astaci* remains unknown. It has been shown that the pathogen prevalence in a weakly infected population decreases from April to November, whereas the prevalence in a highly infected population remains high over several years and during different seasons (Matasová *et al*., 2011).

*Supplementary Transmission Experiment*

The classical transmission experiment conducted for all six populations tested out of the total of nine coexisting populations revealed no indication of an infection with *A. astaci*, and supported thereby the molecular analyses. The power of the experiment was confirmed when noble crayfish was kept with *A. astaci* positive spiny-cheek crayfish from a single population from Plociowe. In this aquarium, the noble crayfish mortality was about three times higher than in the stock with crayfish from coexisting populations. The increased mortality serves as a strong sign of disease transmission (Matthews & Reynolds, 1990; Oidtmann *et al*., 2006) from infected spiny-cheek crayfish to uninfected noble crayfish. Noble crayfish from this aquarium indeed were infected at the end of the experiment. The results of the transmission experiments therefore agree with the qPCR results. However, the long duration of transmission experiments is a disadvantage, particularly if further conservation measures depend on the confirmed infection status with *A. astaci*. Consequently, the fast qPCR verification of a positive case makes the time-consuming transmission experiments redundant. On the other hand, for the verification of pathogen absence, the transmission experiment turned out as a valuable supplementary test. Since a wrong negative result can have fatal consequences for native crayfish, a second independent verification is indeed strongly recommended.

*Conservation and Management Considerations*

Knowledge about the presence of the crayfish plague can influence conservation activities and actions (Kozubíková *et al*., 2009). In Europe, many indigenous crayfish populations have succumbed due to this disease (e.g. Alderman, 1996). The pathogen poses a severe threat to the noble crayfish, and if a highly virulent *A. astaci* genotype infects the coexisting populations all noble crayfish will die within a few weeks. On the other hand, infection by the less virulent As-genotype might be less harmful (Viljamaa-Dirks *et al*., 2011). An important outcome of this study is that European crayfish populations are not necessarily lost, at least not in the short run if American crayfish occur in the same water. In the long run, however, competitive exclusion of native crayfish is a likely outcome (see below). However, since predators, fishing gear and any item that has been in contact with contaminated water, may transmit the pathogen

between waterbodies (OIE, 2009), the risk of disease transmission into pathogen-free waters, and thus also into the coexisting population is great unless measures are taken to prevent infection from outside. At the same time, we cannot be 100% sure that these populations are pathogen free, as there is still a 2% probability of positive carriers in the populations (given the test assumptions made here). Hence, coexisting populations should not be considered as fully safe regarding further transmission risk originating from these populations.

Non-infected European crayfish can be translocated to refuge localities like “ark sites” to conserve intraspecific diversity (Kozák *et al*., 2011; Peay, 2009). Such crayfish should preferably originate from single species lakes of native crayfish. However, native crayfish from well documented plague-free coexisting populations may also be considered worth translocating into ark sites depending on their genetic composition and uniqueness. This is particularly important since permanent coexistence, even in the absence of crayfish plague, seems unlikely in the long run, due to other factors such as competitive exclusion by the alien crayfish, as has been shown for coexistence between noble crayfish and spiny-cheek crayfish (Schulz *et al*., 2006) or noble crayfish and signal crayfish (Söderbäck, 1994; Westman *et al*., 2002).

**ACKNOWLEDGMENTS**

We thank C. Dümpelmann and S. Schleich for providing American crayfish samples and M. Keller for providing noble crayfish from his hatchery in Augsburg. We thank D. Strand and W. Jarausch for assistance in the laboratory. Furthermore, we thank three anonymous reviewers for comments on a previous version of the manuscript. The study was financially supported by the Deutsche Bundesstiftung Umwelt and the University of Koblenz-Landau.

## REFERENCES


Abrahamsson S. (1966) Dynamics of an isolated population of the crayfish *Astacus astacus Linné. Oikos* **17**, 96-107.

Alderman D.J. (1996) Geographical spread of bacterial and fungal diseases of crustaceans. *Revue Scientifique Et Technique - Office International Des Epizooties*, **15**, 603–632.

Bohman P., Nordwall F. & Edsman L. (2006) The effect of the large-scale introduction of signal crayfish on the spread of crayfish plague in Sweden. *Bulletin Français de la Pêche et de la Pisciculture*, **380-381**, 1291-1302.

Dieguez-Uribeondo, J., Cerenius, L., Söderhäll, K. (1995) Physiological adaptations in an *Aphanomyces astaci* strain from the warm-water crayfish *Procambarus clarkii*. *Mycological Research*, **99**, 574–578

Dohoo I., Martin W. & Stryhn H. (2003) Veterinary Epidemiologic Research. AVC Inc, Charlottetown, Prince Edward Island, Canada, pp 47-49.

Holdich D.M. (2002) Distribution of crayfish in Europe and some adjoining countries. *Bulletin Français de la Pêche et de la Pisciculture*, **367**, 611–650.

Holdich D.M., Reynolds J.D., Souty-Grosset C. & Sibley P.J. (2009) A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowledge and Management of Aquatic Ecosystems*, **11**, 394–395.

Huang T.S., Cerenius L. & Söderhäll K. (1994) Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci,* by random amplification of polymorphic DNA. *Aquaculture* **126**, 1-9.

Jussila J., Makkonen J., Vainikka A., Kortet R., & Kokko H. (2011) Latent crayfish plague (*Aphanomyces astaci*) infection in a robust wild noble crayfish (*Astacus astacus*) population. *Aquaculture* **321**, 17-20.

Kokko, H., Koistinen, L., Harlioğlu, M.M., Makkonen, J., Aydın, H. & Jussila, J. (2012) Recovering Turkish narrow clawed crayfish (*Astacus leptodactylus*) populations carry *Aphanomyces astaci*. *Knowledge and Management of Aquatic Ecosystems*, **404**, 1-7.

Kozák P., Füreder L., Kouba A., Reynolds J.D. & Souty-Grosset C. (2011) Current conservation strategies for European crayfish. *Knowledge and Management of Aquatic Ecosystems*, **401**, 01.

Kozubíková E., Filipova L., Kozak P., Duris Z., Martin M.P., Dieguez-Uribeondo J., *et al*. (2009) Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in invasive American crayfishes in the Czech Republic. *Conservation Biology*, **23**, 1204-1213.

Kozubíková E., Petrusek A., Ďuriš Z., Kozák P., Geiger S. Dieguez-Uribeondo J. & Oidtmann B. (2008) The old menace is back: recent crayfish plague outbreaks in the Czech Republic. *Aquaculture*, **274**, 208-217.

Kozubiková, E., Viljamaa-Dirks, S., Heinikainen, S., Petrusek, A. (2011a) Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague pathogen *Aphanomyces astaci*. *Journal of Invertebrate Pathology*, **108,** 214-216.

Kozubíková E., Vrålstad T., Filipová L. & Petrusek A. (2011b) Re-examination of the prevalence of *Aphanomyces astaci* in North American crayfish populations in Central Europe by TaqMan MGB real-time PCR. *Diseases of Aquatic Organisms*, **97**, 113-125.

Lowe S., Browne M., Boudjelas S. & De Poorter M. (2004) *100 of the world's worst invasive alien species. A selection from the Global Invasive Species Database*. The Invasive Species Specialist Group (ISSG), a specialist group of the Species Survival Commission (SSC) World Conservation Union (IUCN), pp 12. First published as special lift-out in *Aliens 12*, 2000. Updated and reprinted version: 2004.

Makkonen J., Jussila, J. & Kokko, H. (2012) The diversity of the pathogenic Oomycete (*Aphanomyces astaci*) chitinase genes within the genotypes indicate adaptation to its hosts. *Fungal Genetics and Biology*, **49**, 635–642.

Matasová K., Kozubíková E., Svoboda J., Jarošík V. & Petrusek A. (2011) Temporal variation in the prevalence of the crayfish plague pathogen (*Aphanomyces astaci*) in three Czech spiny-cheek crayfish populations. *Knowledge and Management of Aquatic Ecosystems*, **401**, 14.

Matthews M. & Reynolds J.D. (1990) Laboratory investigations of the pathogenicity of *Aphanomyces astaci* for Irish freshwater crayfish. *Hydrobiologia* **203(3)**, 121-126.

OIE (Office international des épizooties) (2012) Crayfish plague (*Aphanomyces astaci*). Chapter 2.2.1. In: Manual of Diagnostic Tests for Aquatic Animals 2012, pp. 101-118. Office international des épizooties, Paris, http://www.oie.int/international-standard-setting/aquatic-manual/access-online/. Accessed 9th January 2013.

Oidtmann B., Geiger S., Steinbauer P., Culas A. & Hoffmann R.W. (2006) Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction. *Diseases of Aquatic Organisms,* **72**, 53-64.

Pârvulescu L., Schrimpf A., Kozubíková E., Resino S.C., Vrålstad T., Petrusek A. & Schulz R. (2012) Invasive crayfish and crayfish plague on the move: first detection of the plague agent *Aphanomyces astaci* in the Romanian Danube. *Diseases of Aquatic Organisms*, **98**, 85-94.

Persson, M., Cerenius, L., Söderhäll, K. (1987) The influence of haemocyte number on the resistance of the freshwater crayfish, *Pacifastacus leniusculus* Dana, to the parasitic fungus *Aphanomyces astaci*. *Journal of Fish Diseases*, **10**, 471–477.

Peay S. (2009) Selection criteria for “ark sites” for white-clawed crayfish. In: *Crayfish Conservation in the British Isles*. (Eds J. Brickland, D.M. Holdich, E.M. Imhoff), pp. 63–69. Leeds, UK, 25 March, 2009.

R Development Core Team. (2011) R: a language and environment for statistical computing. *R* Foundation for Statistical Computing, Vienna.

Reynolds J. & Souty-Grosset C. (2012) *Management of freshwater biodiversity: crayfish as bioindicators*. Cambridge University Press. 384pp.

Sala O.E., Chapin F.S., Armesto J.J., Berlow E., Bloomfield J., Dirzo R., *et al*. (2000) Global biodiversity scenarios for the year 2100. *Science*, **287**, 1770-1774.

Schrimpf A., Chucholl C., Schmidt T. & Schulz R. (in press) Crayfish plague agent detected in populations of the invasive North American crayfish *Orconectes immunis* (Hagen, 1870) in the Rhine River, Germany. *Aquatic Invasions*, 8. (available online: http://www.aquaticinvasions.net/2013/accepted.html)

Schrimpf A., Pârvulescu L., Copila-Ciocianu D., Petrusek A. & Schulz R. (2012) Crayfish plague pathogen detected in the Danube Delta – a potential threat to freshwater biodiversity in southeastern Europe. *Aquatic Invasions*, **7(4)**: 503–510.

Schulz H.K., Smietána P., Maiwald T., Oidtmann B. & Schulz R. (2006) Case studies on the co-occurrence of *Astacus astacus* (L.) and *Orconectes limosus* (Raf.): snapshots of a slow displacement. *Freshwater Crayfish*, **15**, 212–219.

Skov C., Aarestrup K., Sivebæk F., Pedersen S., Vrålstad T. & Berg S. (2011) Non-indigenous signal crayfish *Pacifastacus leniusculus* are now common in Danish streams: preliminary status for national distribution and protective actions. *Biological Invasions*, **13**, 1269–1274.

Söderbäck, B. (1994) Interactions among Juveniles of 2 Fresh-Water Crayfish Species and a Predatory Fish. *Oecologia*, **100**, 229-235.

Söderhäll, K. & Cerenius, L. (1992) Crustacean immunity. *Annual Review of Fish Diseases*. **2**, 3–23

Söderhäll K. & Cerenius L. (1999) The crayfish plague fungus: history and recent advances. *Freshwater Crayfish*, **12**, 11–35.

Stevenson M. with contributions from Nunes T., Sanchez J. & Thornton R. (2010) epiR: Functions for analysing epidemiological data. R package version 0.9-27. http://CRAN.R-project.org/package=epiR.

Strand D.A., Holst-Jensen A., Viljugrein H., Edvardsen B., Klaveness D., Jussila J. & Vrålstad T. (2011) Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. *Diseases of Aquatic Organisms*, **95**, 9-17.

Strand, D.A., Jussila, J., Viljamaa-Dirks, S., Kokko, H., Makkonen, J., Holst-Jensen, A., Viljugrein, H., Vrålstad, T. (2012) Monitoring the spore dynamics of *Aphanomyces astaci* in the ambient water of latent carrier crayfish. *Veterinary Microbiology*, **160**, 99-107.

Svoboda J., Kozubíková E., Kozák P., Kouba A., Bahadir Koca S., Diler Ö., *et al*. (2012) PCR detection of the crayfish plague pathogen in narrow- clawed crayfish inhabiting Lake Eğirdir in Turkey. *Diseases of Aquatic Organisms*, **98**, 255-259.

Taugbøl T. (2004) Reintroduction of noble crayfish *Astacus astacus* after crayfish plague in Norway. *Bulletin Français de la Pêche et de la Pisciculture*, **372-373**, 315-328.

Tuffs S. & Oidtmann B. (2011) A comparative study of molecular diagnostic methods designed to detect the crayfish plague pathogen, *Aphanomyces astaci*. *Veterinary Microbiology*, **153**, 343-353.

Unestam T. (1969) On the adaptation of *Aphanomyces astaci* as a parasite. *Physiologia Plantarum*, **22**, 221–235.

Unestam T. (1972) On the host range and origin of the crayfish plague fungus. *Report of the Institute of the Freshwater Research Drottningholm*, **52**, 192–198.

Viljamaa-Dirks S., Heinikainen S., Nieminen M., Vennerstrom P. & Pelkonen S. (2011) Persistent infection by crayfish plague *Aphanomyces astaci* in a noble crayfish population - a case report. *Bulletin of European Association of Fish Pathologists*, **31**, 182-188.

Vrålstad T., Johnsen S., Fristad R., Edsman L. & Strand D. (2011) Potent infection reservoir of crayfish plague now permanently established in Norway. *Diseases of Aquatic Organisms*, **97**, 75-83.

Vrålstad T., Knutsen A.K., Tengs T. & Holst-Jensen A. (2009) A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. *Veterinary Microbiology*, **137**, 146–155.

Westman K. & Savolainen R. (2001) Long term study of competition between two co-occurring crayfish species, the native *Astacus astacus* L. and the introduced *Pacifastacus leniusculus* Dana, in a Finnish lake. *Bulletin Français de la Pêche et de la Pisciculture*, **361**, 613-627

# *ERKLÄRUNG*

Hiermit erkläre ich, dass ich die eingereichte Dissertation

- selbständig verfasst habe und alle wörtlich oder sinngemäß den Schriften anderer Autorinnen oder Autoren entnommenen Stellen kenntlich gemacht und alle von mir für die Arbeit benutzten Hilfsmittel in der Arbeit angegeben habe,

- weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zwecke der Promotion oder eines Prüfungsverfahrens vorgelegt habe.

Landau, 16. Januar 2013 Anne Schrimpf

# ACKNOWLEDGMENTS

This thesis would not have been possible without the support, help and contribution of many people.

First of all I want to express my gratitude to Prof. Dr. Ralf Schulz for the continuous support in the last years. His enthusiasm, his good ideas and the confidence he put in me was the best motivation I can imagine.

Also, I am grateful to my second referee Prof. Dr. Klaus Schwenk.

I want to thank Dr. Holger Schulz who gave me the opportunity to start this PhD project and who inspired me especially in my first months in Landau and Dr. Kathrin Theissinger for discussions and her help with the first part of my thesis (phylogeography of noble crayfish). Many thanks go to my collegue Thomas Schmidt for good ideas and critical and inspiring discussions. Special thanks to Therese Bürgi and Barbara Thronicke for keeping the lab running and Jone Kammerer for administrative support. I thank Dr. Karolina Kolodziej for the good times that we spent together in the lab and in the office.

Further I thank the International Association of Astacology (IAA) and the forum flusskrebse e.V. for great conferences that gave me the opportunity to present and discuss intermediate results, to meet many interesting people and to build up a scientific network.

I want to thank my Romanian colleague and friend Dr. Lucian Pârvulescu who is the most enthusiastic crayfish person I know. I want to thank him for the good times we had in Romania, Germany, France and Austria and also for inviting German students and me to join his field trip with Romanian students in the Carpathian Mountains in Romania. Further I want to thank Prof. Dr. Trude Vrålstad for inviting me twice to the Norwegian Veterinary Institute in Oslo, Norway and the warm welcome in Oslo. I am grateful to Prof. Dr. Adam Petrusek (Charles University in Prague) for his cooperation and inspiring discussions. I thank Prof. Ivana Maguire for her support and her positive attitude. Further, I thank Christoph Dümpelmann and Sascha Schleich for sharing their experiences

from the field and the tireless crayfish sampling and Dr. Max Keller for the honest criticism and inspiring discussions.

I thank Dr. Lucian Pârvulescu, Dr. Holger Schulz, Dr. Tim Schikora, Dr. Florian Blasberg and Thomas Schmidt for comments on earlier versions of this thesis.

I am grateful to my partner Tim Schikora for his support and for his patience during the last years. This thesis benefited from his help with the thesis layout and his technical support. I am lucky and grateful that we share the same passion for science and biology. Last but not least I am grateful to my parents and my sister who always supported me in so many ways and to my grandparents who always believed in me.

The study was partly financially supported by the Bundesministeriums für Ernährung, Landwirtschaft und Verbraucherschutz (BMELV) through the Bundesanstalt für Landwirtschaft und Ernährung (BLE) (Förderkennzeichen: 07BM019). and by the University Koblenz-Landau. The Interdisziplinäres Promotionszentrum (IPZ) of the University Koblenz-Landau provided financial support for the last month of my thesis through the ‘Förderprogramm für Nachwuchswissenschaftlerinnen'.

# CURRICULUM VITAE

## Personal Data

| | |
|---|---|
| Name: | Anne Schrimpf |
| Date & place of birth: | 21th September 1984, Frankfurt am Main, Germany |
| Email: | schrimpf@uni-landau.de |
| Nationality: | German |

## Education and Career

| | |
|---|---|
| Since Aug. 2009 | PhD student at the Universität Koblenz-Landau, Germany Title of Doctoral Thesis: '*DNA-based methods for freshwater biodiversity conservation – Phylogeographic analysis of noble crayfish (Astacus astacus) and new insights into the distribution of crayfish plague*' |
| May – July 2009 | Research Assistent at the Royal Netherlands Institute for Sea Research (NIOZ), Texel, Netherlands |
| May 2009 | University graduation: Diplom der Biologie (ecquivalent of a Master's degree in Biology) with the grade 'Excellent' |
| | Topic of Diploma Thesis: '*Stopover ecology of a long distance migrant - habitat choice of the Afro-Siberian Red Knot Calidris canutus canutus during northward migration*' at the Royal Netherlands Institute for Sea Research, Texel, Netherlands |
| Oct. 2004 – May 2009 | Studies in Biology at the Johannes Gutenberg-Universität Mainz, Germany |
| June 2004 | Abitur (final secondary-school examinations, general qualification for university entrance) |

## ***Internships / International experiences***

| | |
|---|---|
| March/ Aug. 2011 | Research at the Norwegian Veterinary Institute, Oslo, Norway |
| July 2011 | Students adviser of a field excursion to the Carpathian Mountains in Romania |
| June 2010 | Participation in the International School of Conservation Biology (iSCB) of the Croatian Biological Society in Rovinj, Croatia |
| July 2010 | Field trip to Romania and Bulgaria to collect scientific samples |
| Nov. 2009 | Field trip to Ghana in cooperation with the Biodiversity and Climate Research Centre (BiK-F), Frankfurt, Germany |
| June – Dec. 2008 | Diploma thesis at the Royal Netherlands Institute for Sea Research, Texel, Netherlands |
| May/June 2008 | Research on the *RV Navicula* of the Royal Netherlands Institute for Sea Research, German Wadden Sea |
| Aug./Sep. 2007 | Internship at the Schutzstation Wattenmeer e.V. on the island of Föhr, Germany |
| May 2007 | Participation in the field mapping of breeding birds on the island of Mellum, Germany |
| Aug./Sep. 2005 | Internship on the bird protection island Mellum, Germany |

## ***Miscellaneous***

| | |
|---|---|
| Since 2013 | Member of the editorial committee of the journal 'Annals of West University of Timisoara, Series of Biology' |
| Since 2012 | Coopted member of the board of forum flusskrebse e.V. |
| Since 2011 | ERASMUS coordinator between the West University of Timisoara, Romania and the University of Koblenz-Landau, Germany |
| Since Nov 2009 | Membership: International Association of Astacology, forum flusskrebse e.V. |
| Since Oct 2008 | Membership: International Wader Study Group |
| Since Oct 2005 | Membership: Mellumrat e.V. |

## ***Publications***

### English

Schrimpf A, Maiwald T, Vrålstad T, Schulz HK, Smietána P, Schulz R (in press) Absence of the crayfish plague pathogen (*Aphanomyces astaci*) facilitates coexistence of European and American crayfish in central Europe. Freshwater Biology

Schrimpf A, Chucholl C, Schmidt T, Schulz R (2013) Crayfish plague agent detected in populations of the invasive North American crayfish *Orconectes immunis* (Hagen, 1870) in the Rhine River, Germany. Aquatic Invasions, 8

Schrimpf A, Pârvulescu L, Copila-Ciocianu D, Petrusek A, Schulz R (2012) Crayfish plague pathogen detected in the Danube Delta – a potential threat to freshwater biodiversity in southeastern Europe. Aquatic Invasions, 7 (4): 503–510

Pârvulescu L, Schrimpf A, Kozubíková E, Resino SC, Vrålstad T, Petrusek A, Schulz R (2012) Invasive crayfish and crayfish plague on the move: first detection of the plague agent *Aphanomyces astaci* in the Romanian Danube. Diseases of Aquatic Organisms. 98: 85-94

Schrimpf A, Schulz H, Theissinger K, Pârvulescu L, Schulz R (2011) First large-scale genetic analysis of the vulnerable noble crayfish *Astacus astacus* reveals low haplotype diversity of central European populations. Knowledge and Management of Aquatic Ecosystems. 401: 35

Leyrer J, Brugge M, Dekinga A, Evers A, Schrimpf A, Scheiffarth G, Piersma T (2011) Is there a latitudial gradient in macrozoobenthic community composition in the Schleswig-Holstein Wadden Sea? In : Leyrer, J. (2011) Being at the right time at the right place : interpreting the annual life cycle of Afro-Sibirian red knots. PhD Thesis, University of Groningen, Groningen, The Netherlands

Kees CJ, Schrimpf A, Roumay CD (2009) Herring gulls and lesser black-backed gulls as jackdaw predators. Sula 22(1): 37-42

### German

Schrimpf A, Chucholl C, Schmidt T, Schulz R (2012) Krebspesterreger in invasiven nordamerikanischen Kalikokrebsen (*Orconectes immunis*) im Rheineinzugsgebiet entdeckt. forum flusskrebse, 18

Schrimpf A, Schulz R (2012) Die Krebspest: neue Forschungsergebnisse einer altbekannten Seuchenerkrankung bei Flusskrebsen. Fischerei und Gewässer in Rheinland-Pfalz. 2: 22-23

Schrimpf A, Schulz, R (2012) Die Krebspest: neue Forschungsergebnisse einer altbekannten Seuchenerkrankung bei Flusskrebsen. Fischwaid. 4: 6-7

Schrimpf A, Schulz R (2012) Krebspesterreger im Donaudelta – eine potentielle Gefahr für die Biodiversität in Gewässern Südosteuropas! forum flusskrebse, 17: 38-41

Schrimpf A, Schulz HK, Schulz R (2011) Genetische Diversität des Edelkrebses (*Astacus astacus*) im regionalen und internationalen Zusammenhang. 5. Internationales Flusskrebsforum 2011. Tagungsband. pp 33-35

Schrimpf A, Schmidt T, Schulz R (2011) Aktuelle Methoden zum Nachweis des Krebspesterregers und Anwendungsbeispiele. forum flusskrebse, 16: 24-34

## ***Presentations***

Schrimpf A, Maiwald T, Vrålstad T, Schulz HK, Smietána P, Schulz R (2012) Absence of the crayfish plague agent may explain coexisting populations of European and American crayfish in central Europe. International Association of Astacology (IAA). 19th Symposium, Innsbruck, Austria

Schrimpf A, Pârvulescu L, Copila-Ciocianu D, Petrusek A, Schulz R (2012) Crayfish plague pathogen detected in the Danube Delta – a potential threat to freshwater biodiversity in southeastern Europe. International Association of Astacology (IAA). 19th Symposium, Innsbruck, Austria

Piscione M, Schrimpf A, Theissinger K, Rollin X, Cammaerts R, Herman D, Schulz R (2012) Genetic characterization of Belgian noble crayfish populations (*Astacus astacus*). International Association of Astacology (IAA). 19th Symposium, Innsbruck, Austria

Schrimpf A, Maguire I, Pârvulescu L, Schulz HK, Schulz R, Theissinger K (2012b) First large-scale genetic analysis of the vulnerable noble crayfish *Astacus astacus* in Europe International Association of Astacology (IAA). 19th Symposium, Innsbruck, Austria

Schrimpf A, Schulz HK, Schulz R (2011) Genetische Diversität des Edelkrebses (*Astacua astacus*) im regionalen und internationalen Zusammenhang 5. Internationale Flusskrebstagung, Schaffhausen, Swizerland

Schrimpf A, Schulz HK, Polivka R, Dümpelmann C, Schulz R (2010) Conservation of autochthonous noble crayfish stocks. Student Conference on Conservation Science – New York (SCCS-NY), New York, USA

Schrimpf A, Schulz HK, Polivka R, Dümpelmann C, Schulz R (2010) Phylogeographical patterns of the noble crayfish (*Astacus astacus*). European Crayfish: food, flagships and ecosystem services, Poitiers, France

Schrimpf A, Schulz, HK Polivka, R, Dümpelmann C, Schulz R (2009) Phylogeographic patterns of the noble crayfish (*Astacus astacus*) and recommendations for a regional conservation project in Hessen, Germany. Gesellschaft für Ökologie. 40$^{th}$ Anniversary Meeting, Giessen, Germany

Schrimpf A, Leyrer J, Brugge M, Dekinga A, Evers A, Piersma T. (2009) Rastplatzökologie eines Langstreckenziehers – Habitatnutzung des afro-sibirischen Knutts *Calidris canutus canutus* im Schleswig-Holsteinischen Wattenmeer zur Zeit des Frühjahrszuges. 142. Jahresversammlung der Deutschen Ornithologen-Gesellschaft Pörtschach, Austria

Schrimpf A, Leyrer J, Brugge M, Dekinga A, Evers A, Piersma T (2009) Habitat choice of Afro-Siberian Red Knots (*Calidris canutus canutus*) in the Schleswig-Holstein Wadden Sea during northward migration. Annual Wader Study Group Conference 2009, Texel, The Netherlands

Schrimpf A, Schulz HK, Polivka R, Dümpelmann C. (2009) Identification and restoration of autochthonous noble crayfish stocks (*Astacus astacus*) in Hesse, Germany: a regional conservation project linking science and management. Regional European Crayfish Workshop: Future of Native Crayfish in Europe, Písek, Czech Republic