Ph.D. THESIS IN COTUTELLE
THÈSE EN CO-TUTELLE

between / *entre*

CHARLES UNIVERSITY IN PRAGUE
Faculty of Science

and / *et*

UNIVERSITÉ DE POITIERS
Faculté des Sciences Fondamentales et Appliquées

To obtain degrees of Doctor of Natural Sciences from both universities
Pour obtenir le titre de Docteur en Sciences des deux universités

LENKA FILIPOVÁ

Genetic variation in North American crayfish species introduced to Europe and the prevalence of the crayfish plague pathogen in their populations

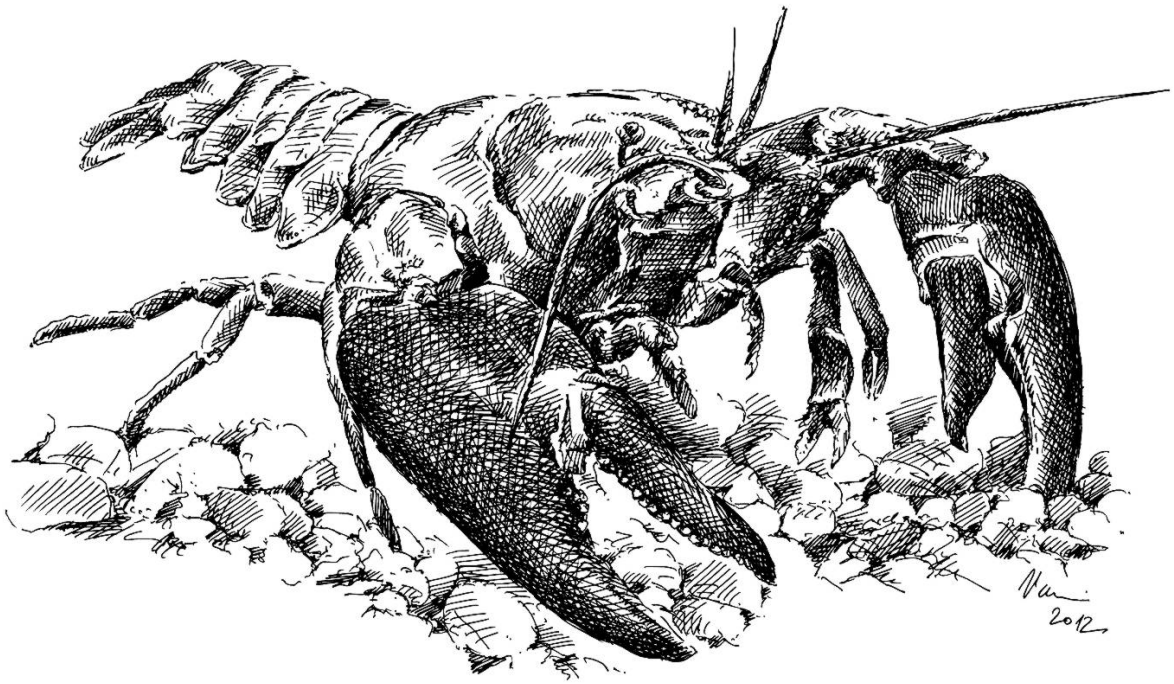
---

Supervisors of the thesis / *Directeurs de thèse*
Petrusek Adam (Charles University in Prague)
Grandjean Frédéric (Université de Poitiers)

Prague, February 2012

I thereby declare that this thesis has not been submitted for the purpose of obtaining the same or any other academic degree earlier or at another institution. My involvement in the research presented in this thesis is expressed through the authorship order of the included publications and manuscripts. All publications and other sources I used when writing this thesis have been properly cited.

In Prague, February 2012 Lenka Filipová



- CONTENTS -

PREFACE AND ACKNOWLEDGEMENTS **1**

Abstrakt (in Czech) **2**

Abstract (in English) **3**

Résumé (in French) **4**

INTRODUCTION **6**

Part 1: Genetic variation in crayfish invaders **10**

Genetic variation in North American crayfish species introduced to Europe 11

Part 2: Crayfish plague **15**

Crayfish plague in France 18

Crayfish plague in Central Europe 19

CONCLUSIONS **20**

FUTURE PERSPECTIVES **21**

REFERENCES **23**

- PUBLICATIONS AND MANUSCRIPTS -

Part 1: Genetic variation in crayfish invaders

CHAPTER I

Filipová L., Lieb D.A., Grandjean F. and Petrusek A., 2011. Haplotype variation in the spiny-cheek crayfish *Orconectes limosus*: colonization of Europe and genetic diversity of native stocks. *Journal of the North American Benthological Society*, 30: 871-881.

CHAPTER II

Filipová L., Kozubíková E. and Petrusek A., 2009. Allozyme variation in Czech populations of the invasive spiny-cheek crayfish *Orconectes limosus* (Cambaridae). *Knowledge and Management of Aquatic Ecosystems*, 394-395, art. no. 10.

CHAPTER III

Filipová L., Grandjean F., Kozubíková E., and Petrusek A. Genetic variation of invasive European populations of signal crayfish, *Pacifastacus leniusculus*. Unpublished manuscript, first draft.

CHAPTER IV

Filipová L., Holdich D.M., Lesobre J., Grandjean F. and Petrusek A., 2010. Cryptic diversity within the invasive virile crayfish *Orconectes virilis* (Hagen, 1870) species complex: new lineages recorded in both native and introduced ranges. *Biological Invasions*, 12: 983-989.

CHAPTER V

Filipová L., Grandjean F., Chucholl C., Soes D.M. and Petrusek. A., 2011. Identification of exotic North American crayfish in Europe by DNA barcoding. *Knowledge and Management of Aquatic Ecosystems*, 401: art. no. 11.

Part 2: Crayfish plague

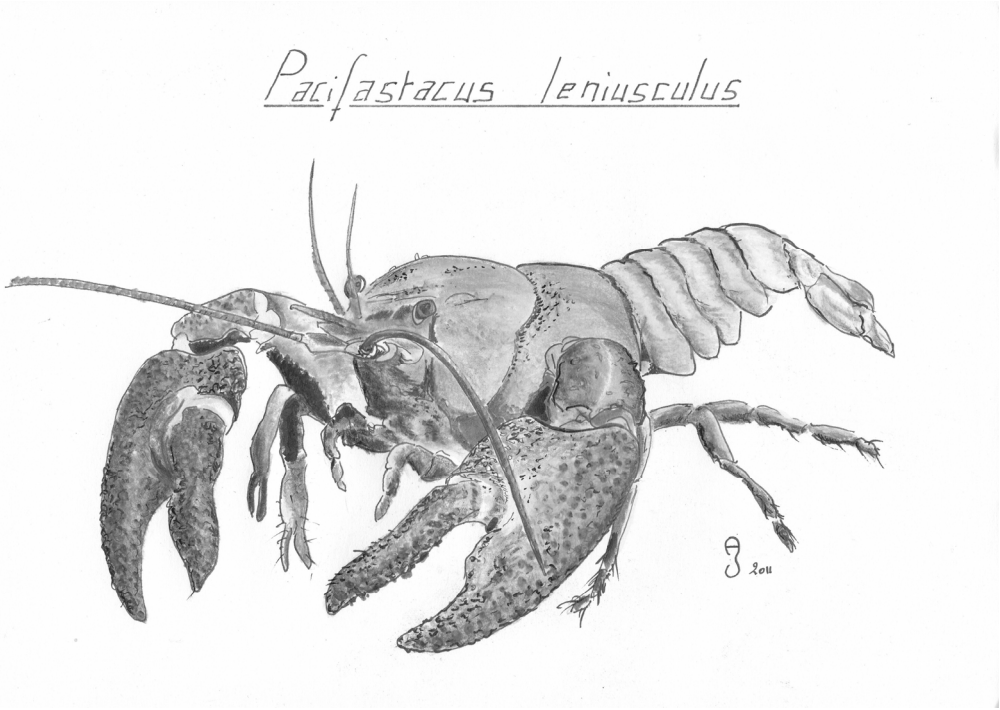
CHAPTER VI

Filipová L., Petrusek A., Matasová K., Delaunay C., Grandjean F. Prevalence of the crayfish plague *Aphanomyces astaci* in populations of signal crayfish *Pacifastacus leniusculus* in France. Unpublished manuscript, first draft.

Appendix

CHAPTER VII

Kozubíková E., Vrålstad T., **Filipová L.** and Petrusek A., 2011. 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.



- PREFACE AND ACKNOWLEDGEMENTS -

My worst marks at high school were from Biology and French Language. If anyone told me in those days that I would spend two years of my life in France studying biology, I would think he is a fool. If this person told me I would dedicate about eight years to studying crayfish, I would recommend him a therapist. Now, after eight years working on crayfish and crayfish plague, partly at the Charles University in Prague and at the University of Poitiers, France, I am very glad for this experience and everything I learned. I had the opportunity to meet many interesting people from the whole world. It was a really exciting time!

I would like to thank everyone who contributed somehow to this thesis and to those who supported me during the last few years. I tried to name at least some of them.

I thank Adam Petrusek for his leading and advices: scientific, technical, theoretical or linguistic, which helped me improve my work significantly. Thus, the present thesis is a result of our fruitful cooperation during the last few years. Furthermore, this dissertation would not be possible without funding from French organisation ONEMA, arranged by Frédéric Grandjean, who supervised my work in the laboratory of Ecology, Evolution and Symbiosis (EES) in Poitiers.

My special thanks belong to Dave Lieb. Our cooperation was arranged solely by email for at least three years. In July 2010 we finally had the opportunity to meet personally and we had a really great time while intensively crayfishing in Pennsylvania.

I would also like to thank Catherine Souty-Grosset, for her valuable comments, advices, provided samples, corrections of French, etc. Similarly, cooperation with Nicolas Poulet was very valuable and pleasant. The technician Carine was my right hand in Poitiers and helped me a lot with laboratory analyses. I also acknowledge members of the laboratory EES, and also Magali Moreau, for their help and support. Moreover, my life abroad was much easier and much more pleasant thanks to my friends and colleagues who I met in Poitiers: Vincent, Maureen, Jessica, Mehdia, Sébastien, Gaël, Benjamin, Samuel, Julien, to name a few. They helped me resolve difficult tasks in the laboratory and, most importantly, they motivated me and helped me relax after long days spent with tubes and pipettes. I will miss you so much.

I am very glad for a nice cooperation with Eva Kozubíková, especially when dealing with problems related to crayfish plague. Klára Matasová survived two months in a laboratory in Poitiers under my leading and analysed sooooo many samples - I appreciate this a lot. My thanks belong also to Eva Hamrová for her help in the laboratory and for her strong support!

Finally, my greatest thanks belong to my parents and my brother, who supported me all the time during my studies, in many ways. I am glad I have such a great family! Huge support came also from my closest friends, Anička and our group of friends, Mikaël, Jitka, Radka and her family, Tomáš, Danča and others.

Many thanks to all of you…

This thesis got its artistic touch thanks to beautiful drawings of crayfish. Děkuji, Mirku! Merci, Julien!

Drawings of crayfish: Miroslav Vomáčka (Vendy Atelier), Julien Amouret

Financial support: French Government, ONEMA (Office National de l'Eau et des Milieux Aquatiques), Grant Agency of the Academy of Sciences of the Czech Republic, Mobility Fund and Grant Agency of the Charles University in Prague

- ABSTRAKT (IN CZECH) -

Biologické invaze korýšů představují vážnou hrozbu pro původní druhy v Evropě. Ve své dizertační práci jsem se zaměřila na nepůvodní sladkovodní raky introdukované do Evropy, a jejich parazita *Aphanomyces astaci*, původce račího moru. Má práce zahrnuje čtyři publikované prvoautorské články (**kapitoly I**, **II**, **IV** a **V**), dva prvoautorské rukopisy (**kapitoly III** a **VI**) a jeden článek, jehož jsem spoluautorkou (**kapitola VII**).

První část (**kapitoly I-V**) je zaměřena na genetickou variabilitu severoamerických raků introdukovaných do Evropy. Ukázali jsme, že genetická variabilita dvou raků, kteří jsou oba úspěšnými invazními druhy v Evropě, se výrazně liší a odráží jejich odlišný způsob kolonizace kontinentu. Rak pruhovaný, *Orconectes limosus*, byl pravděpodobně do Evropy introdukován jen jednou, kdy bylo dovezeno 90 jedinců. Variabilita na úrovni mitochondriální DNA je u raka pruhovaného v Evropě mnohem nižší než v Severní Americe (**kapitola I**), ačkoli určitá míra variability byla zaznamenána na jaderných markerech v jeho středoevropských populacích (**kapitola II**). Opačným příkladem je rak signální, *Pacifastacus leniusculus*, který byl do Evropy introdukován vícekrát, mnoha jedinci. Jeho geneticky vysoce diverzifikované evropské populace patří jedinému poddruhu *P. l. leniusculus* (**kapitola III**). Ten je jedním ze tří poddruhů, které jsou známé ze severní Ameriky. Objev nových linií mitochondriální DNA v severní Americe ovšem ukazuje, že rozdělení těchto poddruhů by mělo být přezkoumáno a bude proto vhodné dále studovat raka signálního v jeho americkém areálu.

Kapitola V ukazuje, že pro přesné určení nově objevených nepůvodních druhů raků v Evropě je vhodná metoda genetického „čárového kódu“ (DNA barcoding). Ověřili jsme identifikaci (určenou na základě morfologie) u některých z těchto invazních raků (*Orconectes juvenilis*, druhový komplex raka *O. virilis*, dále *Procambarus fallax*, a komplex *P. acutus*/*zonangulus*). U studovaných jedinců komplexu kryptických druhů raka *Orconectes virilis* (**kapitola IV**), u raka *O. immunis* a u komplexu *P. acutus*/*zonangulus* jsme nalezli překvapivě vysokou míru genetické variability. Porovnání variability nepůvodních raků v Evropě s daty ze severní Ameriky nám tedy může pomoci odhalit důležité informace o celkové variabilitě v rámci těchto taxonů.

Kapitoly VI a **VII** jsou věnovány detekci račího moru u nepůvodních raků v Evropě. Původce onemocnění, *Aphanomyces astaci* (oomycety), se poprvé objevil v Evropě v roce 1859 a způsobil masový úbytek populací původních druhů raků. Severoameričtí raci přítomní v Evropě mohou tento patogen přenášet a nakazit jím původní evropské druhy; stále tak způsobují úhyny těchto citlivých populací. Informace o promořenosti populací invazních druhů račím morem jsou proto nezbytné, abychom zjistili, jaké nebezpečí tyto populace představují pro původní raky. **Kapitola VI** přináší údaje o promořenosti francouzských populací raka signálního *P. leniusculus* račím morem, které byly získány kvantitativní metodou TaqMan MGB real-time PCR. Potvrdili jsme, že tento druh je ve Francii přenašečem račího moru a doufáme, že naše data přispějí k účinné ochraně původního raka bělonohého, *Austropotamobius pallipes*, v této zemi. V **kapitole VII** jsme použili stejnou metodu detekce *A. astaci*, abychom otestovali vzorky invazních raků ze střední Evropy, které byly dříve zpracovány jinou molekulární metodou. Vysoká citlivost real-time PCR nám umožnila odhalit další nakažené jedince, u kterých nebyla nákaza dříve prokázána. Potvrdili jsme tak, že tato metoda je velmi vhodná pro detekci původce račího moru, přestože je vhodné použít kombinaci více molekulárních metod.

- ABSTRACT (IN ENGLISH) -

Biological invasions by crustaceans represent a serious threat for native species in Europe. In my thesis I focus on non-indigenous freshwater crayfish introduced to Europe and their parasite *Aphanomyces astaci*, the pathogen of the crayfish plague. The thesis consists of four already published first-author papers (**chapters I**, **II**, **IV** and **V**), two first-author manuscripts (**chapters III** and **VI**), and one paper which I co-authored (**chapter VII**).

The first part (**chapters I-V**) focuses on genetic variation in North American crayfish introduced to Europe. We showed that in two crayfish species, both successful invaders in Europe, genetic variation differs significantly, reflecting their different colonization histories on the continent. The spiny-cheek crayfish *Orconectes limosus* was likely introduced to Europe just once, in small numbers (90 individuals). Variation at the mitochondrial DNA (mtDNA) level in the spiny-cheek crayfish in Europe is much lower compared to North America (**chapter I**), although some variation was revealed by nuclear markers in its Central European populations (**chapter II**). In contrast, the signal crayfish *Pacifastacus leniusculus* was introduced to Europe several times, in large numbers. Its European populations are highly diverse genetically and belong to a single subspecies, *P. l. leniusculus*, one of the three subspecies recognised in North America (**chapter III**). Nevertheless, the discovery of new mtDNA lineages in North America showed that the division into subspecies should be revised and more studies from its American range are needed.

Chapter V showed the utility of DNA barcoding, in combination with morphological examinations, for accurate identification of newly established non-indigenous crayfish in Europe. We verified morphological identification of some of these invaders (*Orconectes juvenilis*, *O. virilis* complex, *Procambarus fallax*, *P. acutus*/*zonangulus* complex). Moreover, in studied individuals from the *Orconectes virilis* cryptic species complex (**chapter IV**), *O. immunis*, and the *Procambarus acutus*/*zonangulus* complex surprisingly high variation was found (**chapter V**). Comparing the patterns of variation in non-indigenous crayfish in Europe with data from their American range may therefore reveal important information on overall variation within these taxa.

Chapters VI and **VII** are dedicated to the detection of the crayfish plague pathogen in non-indigenous crayfish in Europe. *Aphanomyces astaci* (oomycetes) first appeared in Europe in 1859 and has substantially reduced native crayfish populations. North American crayfish established in Europe may carry the pathogen and transmit it to indigenous European crayfish, causing mortalities of these susceptible populations that continue today. Information on *A. astaci* prevalence in invasive crayfish populations is therefore necessary for evaluation of the threat they represent to native species. **Chapter VI** provides information on the crayfish plague prevalence in French populations of the signal crayfish *P. leniusculus* obtained by a quantitative TaqMan MGB real-time PCR. We confirm that the species serves as a reservoir of the pathogen in France and we hope our data will contribute to the efficient protection of the native white-clawed crayfish *Austropotamobius pallipes* in the country. In **chapter VII**, the same method of *A. astaci* detection was used to test samples of invasive crayfish from Central Europe which were previously analysed by another molecular method. The high sensitivity of the real-time PCR allowed discovery of infected individuals in populations where the presence of *A. astaci* was not reported before. We therefore confirm that the method is suitable for routine detection of the crayfish plague pathogen, although a combination of molecular methods is recommended.

- RÉSUMÉ (IN FRENCH) -

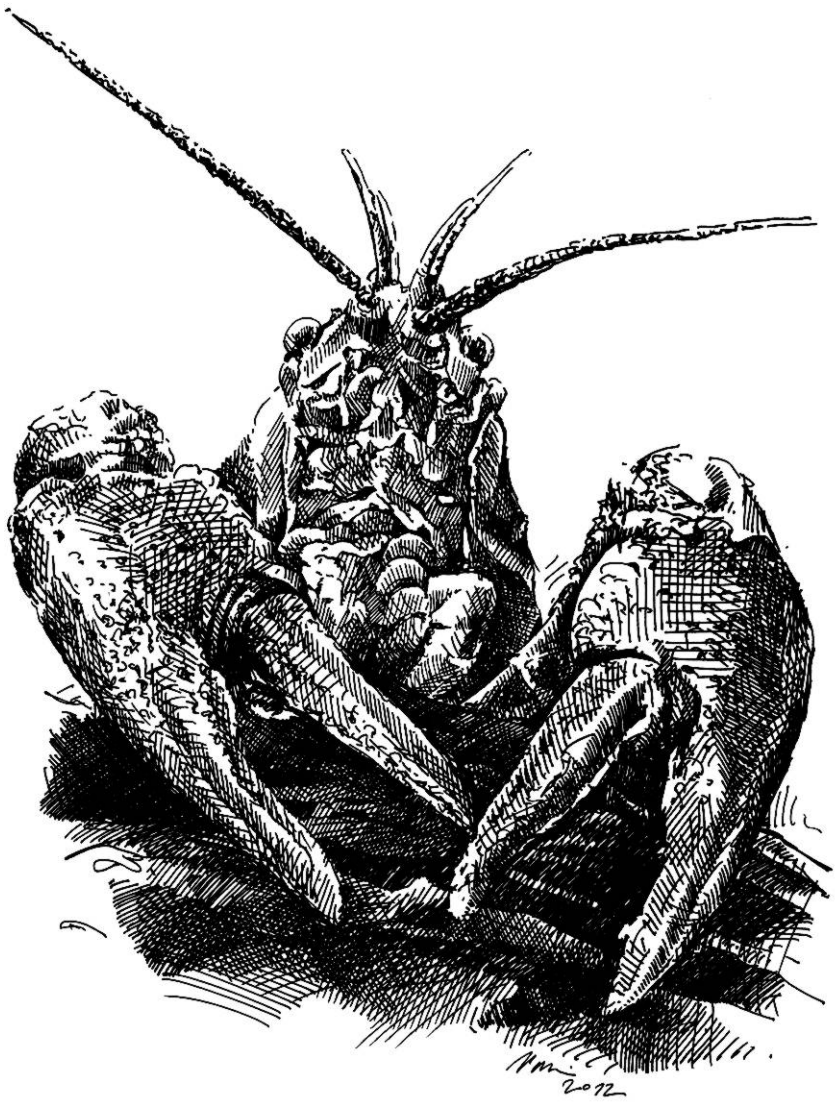
Les invasions biologiques de crustacés représentent une sérieuse menace pour les espèces natives d'Europe. Dans ma thèse je me suis intéressée aux écrevisses non-indigènes introduites en Europe et à leur parasite *Aphanomyces astaci*, le pathogène de la peste de l'écrevisse. La thèse est composée de quatres articles publiés (**chapitre I**, **II**, **IV** et **V**) et de deux manuscrits (**chapitres III** et **VI**) où je suis le premier auteur, et d'un article dont je suis coauteur (**chapitre VII**).

La première partie (**chapitres I-V**) porte sur la diversité génétique des écrevisses d'Amérique du Nord introduites en Europe. Nous avons montré que chez deux espèces avérées invasives, la variabilité génétique varie significativement, ce qui permet de retracer différentes histoires de colonisation sur le continent. L'écrevisse américaine *Orconectes limosus* a été introduite en Europe probablement en une seule fois, avec seulement 90 individus. La variabilité de l'ADN mitochondrial chez cette espèce en Europe est bien inférieure à celle observée en Amérique (**chapitre I**) même si une variation modérée a été mise en évidence par des marqueurs nucléaires dans les populations d'Europe centrale (**chapitre II**). Par contre l'écrevisse de Californie *Pacifastacus leniusculus* a été introduite en Europe en plusieurs fois, avec un grand nombre d'individus. Ses populations européennes, qui sont génétiquement très diverses, appartiennent à une seule sous-espèce *P. l. leniusculus*, une de trois sous-espèces reconnues en Amérique du Nord (**chapitre III**). Mais la découverte des nouveaux clades d'ADN mitochondrial en Amérique du Nord montre que cette division en sous-espèces devrait être révisée et des études supplémentaires sont nécessaires concernant l'aire d'origine en Amérique.

Le **chapitre V** montre que la méthode du code barre génétique (DNA barcoding), en combinaison avec les examens morphologiques, est utile pour une identification exacte des nouvelles espèces d'écrevisses établies en Europe. Nous avons vérifié l'indentification morphologique de certains envahisseurs (*Orconectes juvenilis*, le complexe *O. virilis*, *Procambarus fallax*, le complexe *P. acutus*/*zonangulus*). De plus, une variabilité considérable a été detectée chez le complexe d'espèces cryptiques *O. virilis* (**chapitre IV**), chez *O. immunis*, et le complexe *Procambarus acutus*/*zonangulus* (**chapitre V**). La comparaison des patterns de la variabilité observée chez les écrevisses non-indigènes en Europe avec celle observée dans leur aire de distribution en Amérique peuvent donc apporter des informations importantes sur la variation globale au sein de ces taxa.

Les **chapitres VI** et **VII** portent sur la détection du pathogène de la peste de l'écrevisse chez les écrevisses non-indigènes en Europe. *Aphanomyces astaci* (oomycètes) est apparue en Europe pour la première fois en 1859 et a largement réduit les populations d'écrevisses natives. Les écrevisses d'Amérique du Nord peuvent être porteuses du pathogène et le transmettre aux espèces indigènes en Europe, provoquant encore de nos jours des disparitions de populations entières. Les informations sur le niveau d'infestation par *A. astaci* dans les populations d'écrevisses invasives sont donc nécessaires pour évaluer le danger qu'elles représentent pour les espèces natives. Le **chapitre VI** apporte les informations sur la prévalence de la peste chez l'écrevisse de Californie *P. leniusculus* en France, grâce à la méthode du TaqMan MGB real-time PCR quantitative. Nous confirmons que cette espèce est un réservoir du pathogène en France et nous espérons que ces bases des données contribueront à une protection efficace de l'écrevisse à pattes blanches *Austropotamobius pallipes* dans ce pays. Dans le **chapitre VII** la même méthode de la

détection d'*A. astaci* est utilisée afin de tester les échantillons d'écrevisses invasives d'Europe centrale qui étaient précédemment analysées par une autre méthode moléculaire. Les résultats ont été ensuite comparés pour évaluer le taux d'infestation dans les populations étudiées. La haute sensibilité du real-time PCR nous a permis de découvrir des individus infectés dans des populations où la présence de la peste n'était pas signalée auparavant. Ainsi nous validons que cette méthode est très appropriée pour la détection du pathogène de la peste de l'écrevisse, même si la combinaison de plusieurs méthodes moléculaires est recommandée.



- INTRODUCTION -

Biological invasions have been associated with human presence for thousands of years, but an enormous increase in translocations of organisms around the Earth has occurred in modern times with the rise of transport and commerce (Jeschke and Strayer 2005, Mack et al. 2000, Weijden et al. 2007). Introductions may be intentional or unintentional, with various pathways allowing dispersal of alien organisms in terrestrial as well as aquatic environments (Hulme et al. 2008, Roman and Darling 2007). Introduced organisms then spread to new areas, sometimes with disastrous consequences, both ecological and genetic, on local environments (Mack et al. 2000). Ecological impacts can include direct competition with native species, predation, herbivory, parasitism or mutualism, and indirect habitat changes, indirect competition for resources or changes in trophic interactions. In addition, rapid evolutionary changes may occur in the invader, sometimes followed by accelerated evolution in native species, with possible hybridization and introgression between them (Mack et al. 2000, Sakai et al. 2001). These impacts may lead to the exclusion of native species (Mack et al. 2000). Moreover, the ecological impact of invasions is highly correlated with economic impact (Vilà et al. 2010). According to Ricciardi et al. (2011), biological invasions can even be treated as natural disasters and corresponding precautionary systems should be developed against all invasive species that have the potential for disastrous impacts.

Invading organisms are sometimes slowed down or stopped by various barriers. These may occur during different stages of introduction: importation of the species to a new territory, its introduction into the wild, establishment of a reproducing population and subsequent spread. Barriers may be geographical, preventing the organism from reaching new territories and spreading once there, or reproductional, which do not allow successful establishment of the species (Mack et al. 2000, Weijden et al. 2007). Furthermore, conditions in the invaded environment may not be suitable, e.g., in freshwaters critical factors include temperature, current velocity, water chemistry and abiotic resources, which influence establishment and spread of invasive species (Gherardi 2007).

Multiple introductions of individuals are often necessary for successful establishment and spread of invaders (Weijden et al. 2007). It has been suggested that 10% of imported species become introduced (or feral), 10% of introduced species become established and 10% of these may become pests (“Three Tens Rule”; Williamson 1996). It has become obvious, however, that this rule does not apply in general and that these proportions may vary significantly among taxa and habitats (Jeschke and Strayer 2005, Weijden et al. 2007). Nevertheless, it still seems that species that negatively impact newly invaded territories represent only a small portion of all organisms transported around the Earth. Information on

the different pathways of their spread is therefore essential for the early detection of these invaders, development of methods of prevention, as well as for subsequent management (Hulme et al. 2008).

This thesis deals with invasive North American crayfish in Europe and their parasite, the pathogen of the crayfish plague. In Europe, introductions of non-indigenous freshwater crustaceans represent one of the most striking examples of biological invasions on the continent (Hänfling et al. 2011). The importance of information on the origin, distribution and impact of invasive crustaceans in Europe was highlighted by Holdich and Pöckl (2007). The authors showed that multiple pathways of crustacean invasions to Europe exist, both intentional and unintentional. Intentional introductions are associated with aquaculture, the human and fish food trade, the pet trade, and management and stock enhancement. Among unintentional introductions the authors listed ballast water, fish stockings, fish bait, transport of organisms attached on mobile surfaces or trapped in nets, and transport by predators. The major negative impact of introduced decapods on invaded ecosystems is the loss of macrophytes and some benthic animals, which further influences local food webs (Strayer 2010). Water and sediment characteristics may also be modified significantly, as was shown for the invasive red swamp crayfish *Procambarus clarkii* (Angeler et al. 2001). Some decapods have also caused a decline of native species in parts of the invaded area (Holdich and Pöckl 2007). In Europe, strong negative effects of introduced freshwater crustaceans have been shown for amphipods, such as the Pontocaspian *Dikerogammarus villosus* and the North American *Gammarus tigrinus*, and for invasive crayfish (Holdich and Pöckl 2007).

Transmission of parasites often plays an important role in the exclusion of native species by introduced ones (Mack et al. 2000, Poulin et al. 2010). Nevertheless, the impact of introduced diseases on freshwater ecosystems has probably been underestimated, because little attention has been paid to them compared to human diseases (Strayer 2010). In aquatic environments, the transmission of pathogens from an invader to naïve native species has been demonstrated for fish parasites, including the infectious salmon anemia, the rosette agent *Sphaerothecum destruens*, and *Gyrodactylus salaris*, all of which cause severe mortalities on fish (especially salmonids), and the Asian nematod *Anguillicola crassus* which affects European eels (Gozlan et al. 2009, Peeler and Feist 2011, Spickler 2010). In amphibians, the most striking example is the fungal parasite *Batrachochytrium dendrobatidis*, which has devastated their populations in North and South America, Europe and Australia (Daszak et al. 2003).

An example of such host-switching was reported also for the pathogen of the crayfish plague *Aphanomyces astaci*, originating in North America, and its hosts, native and invasive crayfish in Europe. In 1859, the first native crayfish mortalities attributed to this disease appeared in Lombardy (northern Italy) and in the following years *A. astaci* quickly spread

across Europe, leading to severe losses of indigenous crayfish populations (Alderman 1996). After more than 150 years, the pathogen still represents a serious threat to native crayfish in Europe (Souty-Grosset et al. 2006).

The European native crayfish most affected by crayfish plague are the white-clawed crayfish *Austropotamobius pallipes*, and the noble crayfish *Astacus astacus*. Until the mid-19th century their populations were strong and they were harvested and consumed in large numbers. However, after the first outbreak of the crayfish plague in Italy and especially after the second outbreak in France, numerous native crayfish populations were eliminated by the disease (Souty-Grosset et al. 2006). The stone crayfish *Austropotamobius torrentium* is also highly susceptible to the pathogen. In the Czech Republic, one mass mortality of *A. torrentium* has been recorded in 2005, and at least one more population is assumed to have been lost recently due to crayfish plague (Kozubíková et al. 2008). The overall impact of the disease on European populations of the stone crayfish is not well documented, but it is possible that *A. astaci* has contributed to its decline (Holdich et al. 2003). Some populations of *A. torrentium* in the Danube watershed are located relatively close to localities with invasive signal crayfish *Pacifastacus leniusculus* (in Hungary and Slovakia) and spiny-cheek crayfish *Orconectes limosus* (in Romania). Individuals of these invaders in the area are infected by the crayfish plague pathogen and might therefore have a disastrous impact on local populations of the stone crayfish (Kozubíková et al. 2009, Pârvulescu et al. 2012).

To replace lost native crayfish populations, several non-indigenous crayfish species have been introduced to Europe. The first crayfish intentionally introduced to Europe was *Orconectes limosus*, which was released in 1890 in Pomerania, currently western Poland (Kossakowski 1966). Later, additional non-indigenous crayfish species ("NICS") were introduced. Most were North American crayfish species from the family Cambaridae, but at least three Australian *Cherax* species (Parastacidae) were also brought to the continent (Jaklič and Vrezec 2011, Souty-Grosset et al. 2006). Three North American species, sometimes called "Old NICS", are now particularly abundant and invasive in Europe: the spiny-cheek crayfish *Orconectes limosus* which is present in at least 20 countries, the signal crayfish *Pacifastacus leniusculus* found in at least 25 countries and the red swamp crayfish *Procambarus clarkii* reported from 8 countries, mostly in warmer parts of southwestern Europe (Holdich et al. 2009, Souty-Grosset et al. 2006). Additionally, other non-indigenous crayfish species, or "New NICS", have been introduced to Europe in the last few decades, most likely through the aquarium trade and aquaculture (Holdich et al. 2009, **chapter V**).

North American crayfish negatively affect native European crayfish populations in a variety of ways. In particular, they serve as a reservoir of the crayfish plague pathogen and transmit it to susceptible hosts. Invasive crayfish also affect European species indirectly by altering their environment, but also by direct competition for resources (Holdich 1999).

Together with other factors, such as pollution or habitat alteration by humans, invasive crayfish have contributed to the decline of native crayfish populations. *Astacus astacus*, *Austropotamobius pallipes* and *Austropotamobius torrentium* are now considered endangered species in Europe and they are listed in Appendix III of the Bern Convention as protected species and in the EU Habitats Directive. In contrast, the pathogen of the crayfish plague is listed in the IUCN list of 100 worst invasive alien species in the world (Lowe et al. 2000). Moreover, *Aphanomyces astaci* and the three most widespread invasive crayfish species in Europe, *Procambarus clarkii*, *Orconectes limosus* and *Pacifastacus leniusculus* are considered to be among the worst invaders threatening biodiversity in Europe (DAISIE 2008, EEA 2007).

Efficient management of invaders requires information on the biology of these species, their origin and routes of introduction, and history of colonization (Hulme et al. 2008). To investigate some of these features in invasive populations, molecular tools have been shown to be useful (Sakai et al. 2001). Although this approach has its limitations, it has proven suitable, e.g., in identifying alternative sources of non-native species or evidence of multiple introductions (Blanchet 2012, Fitzpatrick et al. 2011). To address some of these questions concerning North American crayfish invasive in Europe, we analysed their European and American specimens using molecular techniques. These studies comprise the first part of my thesis (**chapters I-V**). In **chapter I** we studied genetic diversity of *Orconectes limosus* individuals from Europe and North America in order to assess whether European populations came from a single source, and tried to identify their origin. Analyses of *O. limosus* from its original range also allowed us to evaluate to what extent losses of threatened local populations may impact overall intraspecific genetic diversity. In **chapter II** we analysed Central European populations of *O. limosus* to evaluate the level of intra- and among-population genetic variation and to relate it to the invasion process. European populations of another species, *Pacifastacus leniusculus*, were studied to test for the presence of currently recognised subspecies in Europe and to investigate variation in these introduced populations (**chapter III**). Finally, we analysed European individuals of introduced North American crayfish and data were compared with those available from North America (**chapter V**). In that study, we focused on “New NICS” and tried to confirm morphological identification of some of the studied species. We also wanted to show the utility of DNA barcoding for identifying crayfish invaders. Preceding that overall study, we focused in detail on European populations of one “New NICS”, identified as the virile crayfish *Orconectes virilis*, to assess their position within other lineages of the *O. virilis* species complex known from its North American range (**chapter IV**). Four studies from this part of the thesis have already been published (**chapters I**, **II**, **IV** and **V**) and one is presented as an unpublished manuscript (**chapter III**).

To protect native crayfish more efficiently, there is a need to evaluate the danger that non-indigenous crayfish populations represent to native species. In Europe, the major conservation issue is the threat these invaders pose to local crayfish by the transmission of the crayfish plague pathogen *Aphanomyces astaci*. Several molecular methods for *A. astaci* detection in crayfish have been developed; the most recent ones are also suitable for the detection in non-indigenous crayfish where the pathogen load is often relatively low. We used one of these methods to analyse *A. astaci* prevalence in French populations of the invasive signal crayfish *Pacifastacus leniusculus* (**chapter VI**), and to evaluate the danger these populations represent for native crayfish, especially the white-clawed crayfish *Austropotamobius pallipes*. I also contributed to studies that analysed the prevalence of *A. astaci* in Central European populations of two invasive crayfish, *O. limosus* and *P. leniusculus* (Kozubíková et al. 2009 and **chapter VII**). The study that focuses on the situation in France is presented as a manuscript; the paper re-assessing the prevalence of *A. astaci* in Central Europe, of which I am a co-author, has already been published.

Most of the non-indigenous crayfish research in Europe has focused on characteristics of colonization of new areas by these species, on their ecology, behavior, interactions with native species and possible ways to eradicate invasive populations (Souty-Grosset et al. 2006). However, little was known about their genetic variation, in Europe as well as in North America. I have therefore dedicated a substantial part of my PhD studies, and the first part of this thesis, to that subject. In the second part, I contributed to the recent boom of studies that took advantage of the development of molecular methods to detect the crayfish parasite *Aphanomyces astaci*. On the following pages, I will discuss these two topics in more detail.

PART 1: GENETIC VARIATION IN CRAYFISH INVADERS

Knowledge of genetic diversity and evolutionary processes in invasive species is important when studying biological invasions. In invading populations, genetic variability is influenced by the history of native populations of the species and characteristics of its introduction to the area, both historical and demographical (Estoup and Guillemaud 2010). For a long time scientists supposed that invasive populations had reduced genetic variation due to colonization bottlenecks. However, it was recently shown that different factors such as high propagule pressure and multiple introductions may prevent the irreversible loss of genetic variation during introduction (Roman and Darling 2007). Moreover, reduced variation in introduced populations of some organisms does not necessarily mean an obstruction for their success when colonizing new habitats, as has been shown for various organisms, e.g.,

the guppy *Poecilia reticulata* and greyling *Thymallus thymallus* (Roman and Darling 2007). In an extreme case, colonization by a single genotype may be extremely successful, as has been documented for an asexual American water flea clone (*Daphnia pulex* x *pulicaria* hybrid) in Africa (Mergeay et al. 2006).

To better understand the process of invasion, it may therefore be useful to evaluate the amount of genetic variation lost during the introduction of the studied species and to determine if multiple introduction events occurred (Barbaresi et al. 2007). In some cases, results from an invaded area should be compared with data from the original range of the species. If these data are not available, sampling and analyses of native populations often follow, sometimes with unexpected results. Studying non-indigenous species may therefore provide important information on populations in their native range. When variation in introduced and native populations of the species is analysed, we may also learn more about the patterns of diversity within the studied taxon in general (**chapter V**). In certain cases, presence of cryptic species in the invaded area may be uncovered (Bickford et al. 2007, **chapter V**). In my thesis I focused on North American crayfish present in Europe. These species differ in their colonization history, number of introduction events and number of introduced individuals. North American crayfish therefore provide an interesting model for studies of biological invasions in Europe.

Genetic variation in North American crayfish species introduced to Europe

In 2005 when I began studying non-indigenous crayfish in Europe, only a few studies of genetic diversity in their introduced populations were available (Agerberg 1990, Agerberg and Jansson 1995, Barbaresi et al. 2003, Grandjean and Souty-Grosset 1997). Since then, I have substantially contributed to knowledge of variation in these taxa, and brought new information on their identity, taxonomical status, population structure and variation in their invaded and native ranges.

Genetic variation of the spiny-cheek crayfish ***Orconectes limosus*** in North America has not been studied before. This species received relatively little attention in its native range until recently, although it is endangered in some regions, particularly in Pennsylvania and Maryland (Lieb et al. 2011), and has probably been completely extirpated by other crayfish species in West Virginia (Swecker et al. 2010). Thus, information on variation in its endangered populations is important for the preservation of rare haplotypes and future conservation management of the species (**chapter I**). Genetic variation in European *O. limosus* populations has not been analysed either. The success of the species in Europe and quick colonization of new habitats seemed surprising given that the literature indicated that a low number of individuals had been imported just once to Europe. If unreported

multiple introductions occurred, this might have assured that more variation was brought to Europe from the original range. If the species is highly structured in its original range, multiple introductions can even lead to higher variation in introduced than native populations (e.g. Kolbe et al. 2004, Sakai et al. 2001). In **chapter I** we therefore investigated genetic variation in *Orconectes limosus* and looked for potential signs of multiple introduction events from North America to Europe. We also sought to determine if its source area could have been in the basin of the Delaware River (on the border between Pennsylvania and New Jersey, eastern USA), as suggested by Schikora (1916). Analyses of genetic variation in invasive but also in native American *O. limosus* populations were therefore needed to understand the invasion process of the species. Variation in European populations was much lower than that found in North America. Despite an apparent bottleneck effect during introduction and low variation in the species in Europe, the invasion success of *O. limosus* does not seem to be significantly reduced (**chapter I**). Still, substantial variation in nuclear markers was maintained during its introduction, as was shown in **chapter II** and in another study that tested variation of several microsatellite loci (Hulák et al. 2010), to which I also contributed. The successful spread of the species in Europe and its high invasiveness might have also been facilitated by its reproductive plasticity: as recently demonstrated, *Orconectes limosus* is capable of facultative parthenogenetic reproduction (Buřič et al. 2011). However, more studies are needed to confirm or reject this hypothesis.

In the signal crayfish ***Pacifastacus leniusculus***, three subspecies are recognised in North America: *Pacifastacus l. leniusculus*, *P. l. trowbridgii* and *P. l. klamathensis* (Miller 1960). They are difficult to distinguish because their morphological characters as well as their range of distribution overlap. Sonntag (2006) analysed signal crayfish populations mainly from the Klamath River Basin in California and Oregon in North America and demonstrated that the three subspecies are distinguishable at the mtDNA level. However, the range of distribution of signal crayfish in North America is much larger than the area studied by the author. Recent analyses of samples from other regions in the Pacific Northwest (western part of the USA and Canada) uncovered the presence of new mtDNA lineages and showed that variation within signal crayfish is higher than previously suggested (**chapter III**, E.R. Larson, pers. comm.). The taxonomic status of the three subspecies was also questioned, as genetic divergences between them are surprisingly high, suggesting that they may be separate species (Sonntag 2006). More information on the distribution, reproductive compatibility, and genetic variation of major signal crayfish lineages in North America are therefore needed to resolve these questions. Although invasive in Europe, Japan and partially in northwestern USA, *Pacifastacus leniusculus* may be endangered in some parts of its North American native range by other crayfish species and habitat modifications (Bondar et al. 2005, Larson and Olden 2011), which is similar to the situation in the spiny-cheek crayfish *Orconectes*

limosus (Lieb et al. 2011, **chapter I**). In the Rogue River system in Oregon, *P. l. klamathensis* has been replaced by *Orconectes neglectus* in most habitats (Bouchard 1977). Investigations of genetic variation within *P. leniusculus* may therefore help protect its unique evolutionary lineages.

In Europe, genetic variation in *P. leniusculus* has been little studied so far, and only a few populations from restricted areas have been analysed. Using allozyme electrophoresis, Agerberg (1990) and Agerberg and Jansson (1995) studied variation in Swedish populations of signal crayfish and asked if one or more subspecies are present within them. Although their data suggested the presence of *P. l. leniusculus* and *P. l. trowbridgii*, the third subspecies *P. l. klamathensis* did not seem to be present (Agerberg and Jansson 1995). Based on the RFLP analysis of mtDNA, Grandjean and Souty-Grosset (1997) also suggested that the high variation found in three French signal crayfish populations could reflect the presence of more subspecies in Europe. However, our study (**chapter III**) based on the mtDNA analysis of signal crayfish coming from 17 European countries showed that only the lineage corresponding to *P. l. leniusculus* is found in Europe, although the presence of other subspecies in restricted unsampled areas cannot be excluded. European signal crayfish populations were highly diverse but showed no obvious geographical pattern, which corresponds to colonization of Europe by many individuals of this species, with numerous secondary translocations (**chapter III**).

Although I did not focus on genetic variation in the red swamp crayfish ***Procambarus clarkii*****,** it is interesting to compare the patterns in this species with those discussed above. Although it is an important aquaculture species, *Procambarus clarkii* has not been well studied in North America. Busack (1988) analysed nine populations from the southern USA using allozyme markers and found little variation within the species, suggesting its recent expansion in the area. Much more attention has been given to the species in China where *P. clarkii* is cultured in farms but has also spread into the wild, posing a threat to local ecosystems (e.g. Cao et al. 2010, Yue et al. 2008, 2010). Yue et al. (2008) suggested that parthenogenesis may occur in this species as they found putative clones of *P. clarkii* in studied Chinese populations. Recent comparisons of mtDNA variation in invasive *P. clarkii* populations in Mexico and Costa Rica with variation in the native US range showed association of some of these invasive and native haplotypes, which supports the general belief that this crayfish has relatively low intraspecific variation (Torres and Álvarez 2011). Since little variation was detected by allozyme, COI, or 16S markers in previous studies, variable markers, such as RAPD used in the past, and microsatellite loci analysed in more recent studies, provide better insight into the variation within the species (Barbaresi et al. 2007, Belfiore and May 2000, Zhu and Yue 2008). Some European populations of *P. clarkii* have also been analysed genetically. Red swamp crayfish were first introduced to the

continent from Louisiana to Spain in 1973 in large numbers (40 000 individuals) (Henttonen and Huner 1999). Barbaresi et al. (2003, 2007) showed that the high genetic diversity of European *P. clarkii* populations revealed by RAPD and microsatellite markers may correspond to the scenario of multiple introductions of individuals in its European range from different source locations.

The systematics of **other North American crayfish species** recently introduced to Europe is often unclear and their identification in Europe has been problematic. Morphological examinations often fail due to the existence of cryptic species, lack of characteristic morphological features or simply due to human error resulting from insufficient taxonomic experience. In some cases, genetic studies may reveal erroneous identification of new invaders or the presence of new introduced species or cryptic lineages. In addition to common invaders listed above ("Old NICS"), we analysed representatives of all North American "New NICS" present in Europe: *Orconectes juvenilis*, the *O. virilis* species complex, calico crayfish *O. immunis*, marbled crayfish *Procambarus fallax*, and the white river crayfish complex *P. acutus*/*zonangulus* (**chapter V**). In some of them (*O. juvenilis*, *O. virilis* complex, *Procambarus fallax*, *P. acutus*/*zonangulus* complex), results of DNA barcoding confirmed previous identifications based on morphological features. Moreover, surprisingly high levels of genetic variation were observed when European samples of *O. virilis*, *O. immunis* and *Procambarus acutus*/*zonangulus* complex were compared with reference data from North America.

For one of the "New NICS", the virile crayfish ***Orconectes virilis***, it has been well demonstrated that it represents a cryptic species complex with several species known from North America (Mathews et al. 2008, Mathews and Warren 2008). In this crayfish as well as in *O. limosus*, genetic variation in American populations seems to reflect post-glacial colonization (Mathews et al. 2008, B. Williams, unpublished results; **chapter I**). In our study, we discovered two distinct *O. virilis* lineages that were previously unknown from its American range, one in America, the second in Europe (**chapter IV**), suggesting much higher variation within the complex. Although we did not have enough data to speculate about the origin of the "European" lineage (established in the UK and the Netherlands), recent results show that the two haplotypes detected in this lineage occur in Kansas (B. Williams, pers. comm.), but they might possibly be more widespread.

PART 2: CRAYFISH PLAGUE

Invaders usually have fewer parasites in newly colonised areas than in their original range (Torchin et al. 2003). However, due to the introduction of parasites together with invasive hosts, emerging diseases connected with biological invasions may occur, having a devastating impact on naïve native species (Peeler and Feist 2011, Poulin et al. 2010). Crayfish are known to serve as hosts for a variety of parasites, including the crayfish plague (caused by the saprolegnious oomycete *Aphanomyces astaci*), other *Saprolegnia* species, the microsporidian *Thelohania contejeani* causing the porcelain disease, members of the genus *Psorospermium* (belonging to mesomycetozoea), and several bacterial and viral diseases (Longshaw 2011). Nevertheless, the crayfish plague pathogen *A. astaci* obtained most attention in Europe due to its rapid and immense spread throughout the continent and its severe impact on local crayfish.

When crayfish plague first appeared in Italy in 1859, the cause of the disease was not known. It was identified much later, in 1903, when Schikora recognised that an oomycete (named afterwards *Aphanomyces astaci*) was responsible for plague outbreaks. The source of the pathogen which caused the first European outbreak was, and still remains, unclear. The American origin of *A. astaci* is, however, undisputed. It is supported by the fact that North American crayfish are almost immune to the disease, which is probably a result of a long-term co-evolution of the parasite and its host (Edgerton et al. 2004, Unestam 1973). Furthermore, a specific strain of *A. astaci* has been isolated from an American population of *P. leniusculus* (Huang et al. 1994).

It is now assumed that an American crayfish carrying *A. astaci* was unintentionally introduced to Europe in the mid-19th century (Westman 2002), without having become established in Italian waters. However, the pathogen was transmitted to the native European crayfish. Infection by crayfish plague is usually lethal for these, although recent research shows that in some cases coexistence of native crayfish with the pathogen is possible (Jussila et al. 2011, Pârvulescu et al. 2012, Viljamaa-Dirks et al. 2011), even for decades (Harlioğlu and Harlioğlu 2009, Svoboda et al. 2012). Apart from European crayfish, the disease is also lethal for Australian crayfish species (Unestam 1975) and it is generally assumed that all crayfish, other than those of North American origin, are susceptible. North American crayfish species may die from the plague when severely stressed or exposed to high concentrations of the pathogen (Cerenius et al. 1988, Diéguez-Uribeondo and Söderhäll 1993).

Only asexual reproduction was documented for this parasite. Its basic reproductive cycle (reviewed, e.g., in Cerenius et al. 1988) consists of a release of primary spores in the water from sporangia, their transformation into bi-flagellate zoospores and attachment on the

cuticle of a crayfish. In susceptible species the parasite then penetrates mostly into uncalcified parts of the cuticle and starts growing. It may later affect internal organs, leading to paralysis and eventually death of the infected crayfish. In the final phase *A. astaci* forms zoosporangia which produce primary cysts, being then transformed into secondary zoospores and the cycle is completed (Cerenius et al. 1988, Diéguez-Uribeondo et al. 2006, Vogt 1999). The cycle may be extended with a phase of "repeated zoospore emergence". When a zoospore encysts on substrates other than crayfish, the cyst changes into a zoospore stadium again and continues searching for a suitable attachment surface (Cerenius and Söderhäll 1984). Crayfish plague may be transmitted with infected crayfish, but its zoospores can also be transported by water, on wet objects such as fishing gear, waders etc. (Neveu 2002). Similarly, animals, particularly fish feeding on infected crayfish, may transport the pathogen from an infected to a healthy population (Alderman et al. 1987, Oidtmann et al. 2002). This significantly complicates the management of the disease.

After the first European outbreak in Italy and a second large outbreak of the disease at Plateau des Langres, France, in 1874 (Alderman 1996), the pathogen of the crayfish plague has quickly spread across Europe. Its expansion has been facilitated by frequent translocations of crayfish due to intense trade (Souty-Grosset et al. 2006, Unestam 1973). This was not the only introduction of the pathogen to Europe, as *A. astaci* was most likely brought again several times together with different North American crayfish species. Molecular analyses revealed the presence of at least five groups (A-E) of *A. astaci* genotypes in Europe which were introduced from North America (Kozubíková et al. 2011, Oidtmann et al. 1999), possibly showing various levels of virulence (Diéguez-Uribeondo et al. 1995, Huang et al. 1994, Jussila et al. 2011). Genotypes isolated from mass mortalities of European native crayfish, presumably those originally introduced to Europe, belong to group A. Groups B and C are associated with signal crayfish, while the red swamp crayfish is known to be carrying genotype from group D (Diéguez-Uribeondo et al. 1995, Huang et al. 1994). A recently discovered strain (group E) of the pathogen was detected in the spiny-cheek crayfish (Kozubíková et al. 2011).

Due to the quick removal of moribund and dead animals by predators and scavengers or due to isolation and inaccessibility of localities, outbreaks of diseases in wild animals may remain unnoticed until they reach extreme levels (Plowright 1988). In crayfish, mortalities may remain unreported, as stakeholders and the public, e.g., scuba divers or amateur naturalists, sometimes lack information on the disease (Kozubíková et al. 2008). Due to this ignorance, mortalities of organisms might also be attributed to other causes, such as chemical pollution (Plowright 1988). Mortalities of native crayfish in Europe caused by the crayfish plague occasionally appear in many parts of the continent (Souty-Grosset et al. 2006). The spread of the pathogen is facilitated by the presence of North American crayfish

in Europe, which serve as a reservoir of the disease and may transmit the pathogen to susceptible species. This shows the importance of *A. astaci* detection in these crayfish, as such data may reveal which areas represent the highest danger for native crayfish populations and where conservation efforts should be focused.

Various methods used to detect the pathogen are available. Morphological examinations are complicated, as the species lacks sexual reproductive organs which are used in the identification of other closely related species (Oidtmann et al. 2002). Molecular methods for *A. astaci* detection substantially facilitated the analysis of crayfish without apparent plague symptoms (Oidtmann et al. 2002) and various techniques which do not require the cultivation step have therefore been developed, including amplification of ITS fragments with presumed specific primers (Oidtmann et al. 2006), or a real-time PCR-based technique targeting GH18 chitinase family genes (Hochwimmer et al. 2009). Vrålstad et al. (2009) recently developed a TaqMan minor groove binder real-time PCR targeting the ITS region of the pathogen nuclear ribosomal DNA. This method, which also allows quantifying the amount of *Aphanomyces astaci* DNA in the studied sample, is now considered more sensitive and more specific relative to other available techniques (Tuffs and Oidtmann 2011, **chapter VII**). Furthermore, detection of pathogen spores directly from water samples is now possible, which may aid in the conservation and management of native crayfish in Europe (Strand et al. 2011).

To date, several authors have tested for the presence of *Aphanomyces astaci* in non-indigenous crayfish in Europe by molecular methods, although in most cases, few populations were analysed. The pathogen was detected in the invasive crayfish *Orconectes limosus* from the Czech Republic (Kozubíková et al. 2009, **chapter VII**), Hungary (Kozubíková et al. 2010) and Romania (Pârvulescu et al. 2012). In *Pacifastacus leniusculus* the presence of the plague was confirmed in populations from the Czech Republic (Kozubíková et al. 2009, **chapter VII**), Hungary (Kozubíková et al. 2010), Norway (Vrålstad et al. 2011) and Sweden (Huang et al. 1994, Vrålstad pers. comm.). We supposed that the pathogen could be present in French populations, and our results in **chapter VI** confirm that signal crayfish infected by *A. astaci* are indeed frequent also in France. On the other hand, *A. astaci* was not detected in a recently established population from Denmark (Skov et al. 2011), although low levels of infection cannot be ruled out (see **chapter VII**). The pathogen was also confirmed by molecular approach in *Procambarus clarkii* from Italy (Aquiloni et al. 2011), and we detected *A. astaci* in one of two analysed individuals of this species from France (**chapter VI**).

Although *Aphanomyces astaci* has a strong negative impact on native crayfish, the high virulence of the pathogen may not be entirely negative and may be useful for eradicating susceptible non-indigenous crayfish in Europe. In the Navarra region of Spain,

the crayfish plague pathogen from infected *P. leniusculus* was used to eliminate populations of the Australian crayfish *Cherax destructor*. When individuals of *C. destructor* were infected in the laboratory and released to a pond with healthy specimens, the population was eliminated within several weeks (Souty-Grosset et al. 2006). However, if not done correctly, such use of a virulent pathogen outdoors may represent a serious threat for native crayfish (Scalici et al. 2009) and specific conditions must therefore be fulfilled to avoid its further spread.

Crayfish plague in France

In France, several North American crayfish species are present and may serve as reservoirs of crayfish plague. These are signal crayfish *Pacifastacus leniusculus*, red swamp crayfish *Procambarus clarkii*, spiny-cheek crayfish *Orconectes limosus* and the much less common *O. immunis* and *O. juvenilis*. Although the spiny-cheek crayfish *O. limosus* is the most abundant of these species in France, the signal crayfish *P. leniusculus* is often suspected to be associated with native crayfish mortalities (Bramard et al. 2006, Collas and Salek 2002, Neveu 2002). The signal crayfish is a known carrier of the crayfish plague and it may often come into contact with the native white-clawed crayfish *Austropotamobius pallipes* because they occupy similar habitat (Collas et al. 2007). *Pacifastacus leniusculus* was first brought to France in 1972 from Sweden and two years later to the central part of France from lakes Tahoe and Donner, USA (Arrignon et al. 1999). After its introduction to France, the species quickly spread across the country, being intentionally transported by humans (Neveu 2002).

The white-clawed crayfish is the most abundant native crayfish species in France. However, the number of French populations has significantly decreased in recent decades. Crayfish plague is one of the most important causes of this decline, together with pollution, habitat degradation and intensive fishing and exploitation (Bramard et al. 2006).

No data on the presence and prevalence of *Aphanomyces astaci* in invasive crayfish in France have been available prior to this study. Due to the possible connection between signal crayfish presence and native crayfish mortalities, we focused our research on this species. The aim of our study (**chapter VI**) was to evaluate the prevalence of *A. astaci* in French populations of signal crayfish using the real-time PCR. Our results showed that 103 out of 513 analysed individuals (20%) were infected by the parasite. In Lake Geneva, which still provides signal crayfish for further introductions around the country, 30% of tested crayfish were infected. Although signal crayfish in France serve as a reservoir of the pathogen and may transmit it to native species, other non-indigenous species may also contribute to the spread of the disease. Our preliminary results show that some of the tested

individuals of *O. immunis* and *Procambarus clarkii* from France also harbour the pathogen of the plague (**chapter VI**). More studies of *A. astaci* prevalence in these invasive crayfish would be beneficial as such data are crucial for the conservation of native species in France.

Crayfish plague in Central Europe

Conventional semi-nested PCR (Oidtmann et al. 2006) has been used by a variety of authors to test for the presence or absence of the crayfish plague pathogen in crayfish, e.g., by Kozubíková et al. (2009), Cammà et al. (2010) and Viljamaa-Dirks et al. (2011). This method combined with sequencing seemed suitable for the detection of *Aphanomyces astaci* and in comparison with previous techniques it was not sensitive to closely related species *A. frigidophilus* and *A. invadans* (Oidtmann et al. 2006). When compared to single-round PCR (Oidtmann et al. 2006), semi-nested PCR seemed to be more sensitive; however, the smaller proportions of samples testing positive might have also been influenced by false positives or may have resulted from a degradation of isolates after a long-term storage (E. Kozubíková, unpublished data).

The aim of the study presented in **chapter VII** (the appendix) was therefore to re-evaluate *A. astaci* prevalence in invasive *O. limosus* and *P. leniusculus* from Central Europe previously obtained by this method (Kozubíková et al. 2009) using TaqMan real-time PCR (developed by Vrålstad et al. 2009). In comparison to conventional PCR which showed that 23% of tested crayfish were infected, real-time PCR showed an infection rate of 32% and infected individuals were found in ten additional populations. In most cases these new detections were from *P. leniusculus* populations where the infection rate increased from 3% to 21%. Results of the real-time PCR largely confirmed those obtained by the semi-nested PCR, moreover, the higher number of samples testing positive by the real-time PCR indicated that this method has a higher sensitivity. One false positive case uncovered in the previous study based on conventional PCR, probably representing an undescribed *Aphanomyces* species (Kozubíková et al. 2009), would not be detected as such when analysed by the real-time PCR. This also suggests that the real-time PCR protocol used by us is more sensitive than conventional PCR.

- CONCLUSIONS -

The introduction of non-indigenous crayfish to Europe and co-introduction of crayfish plague are an interesting example of ”parasite-mediated competition“. Although other factors may facilitate their invasion, the interactions of hosts (North American crayfish in our case) with the pathogen of more susceptible species may further increase the success of these invaders (Poulin et al. 2010). It is therefore necessary to study the whole system, not only the invasive host species but also their parasites, as such host-parasite association may have amplified negative effect on invaded ecosystem, leading sometimes to the extinction of naïve species (Cunningham et al. 2003). In the present thesis I examine both hosts and their parasite.

In the first part of the thesis I showed that molecular analyses of European populations of North American crayfish may provide interesting insights into the identity of these invaders, their genetic variation and its relation to the processes during invasion, and the colonization history of studied species in Europe and in America.

Given the difficulties with morphology-based identification of newly introduced crayfish in Europe, molecular methods may be extremely useful. DNA barcoding which was used to verify the identity of “New NICS” in Europe (**chapter IV**) proved to be suitable for fast and accurate identification of newly discovered crayfish, especially in combination with morphological examinations. Moreover, we demonstrated that studying the genetics of introduced populations and comparing these results with newly obtained genetic data from the native range of these invaders may reveal useful general information on variation within the studied taxon. In several cases, analyses of North American crayfish species from their introduced and native ranges showed that their variation is higher than expected (**chapters III**, **IV** and **V**). In *O. immunis* and *P. acutus/zonangulus*, this suggests the existence of species complexes.

In two “Old NICS”, *Orconectes limosus* (**chapter I**) and *Pacifastacus leniusculus* (**chapter III**), the comparison of genetic variation in their invasive and native populations showed that although colonization histories and genetic variation maintained during their introduction to Europe differ significantly, both species may be successful invaders. The study of *O. limosus* populations (**chapter I**) showed that even a single introduction of a relatively low number of individuals may represent a serious threat to the invaded ecosystem. This must be taken into consideration when dealing with new invaders. Furthermore, our results (**chapter I**) show that low variation in introduced populations of an invader does not always hinder its success in colonizing new territories. In contrast, high variation was found in European populations of signal crayfish which was introduced several times in large numbers (**chapter III**). Our finding that all studied European individuals belong

to a single subspecies *P. l. leniusculus* contrasts with previous studies (Agerberg and Jansson 1995, Grandjean and Souty-Grosset 1997) that suggested the presence of more subspecies in Europe. However, the taxonomic status of the three known subspecies is not clear (Sonntag 2006) and our analysis of samples from North America showed that the taxon is more variable genetically than suggested previously (**chapter III**).

In the second part of my thesis, **chapter VI** provides for the first time information on the prevalence of *Aphanomyces astaci* in signal crayfish populations in France. The use of TaqMan real-time PCR (Vrålstad et al. 2009) permitted us to quantify the pathogen load in each analysed crayfish and subsequently evaluate *A. astaci* prevalence in studied populations. We have also determined which of these populations represent the greatest danger for native crayfish due to their high infection ratio. It must be kept in mind that the real agent levels may have been somewhat underestimated because only certain parts of crayfish body were analysed, only a limited number of individuals per population were tested and just a part of the isolate was used in the PCR. However, our results provide the first information regarding the crayfish plague prevalence in France and may be applied in the management of native crayfish in this country, especially the white-clawed crayfish *Austropotamobius pallipes*.

The highly sensitive and specific quantitative real-time PCR allowed us to re-evaluate data on *A. astaci* presence in samples of invasive crayfish *O. limosus* and *P. leniusculus* from Central Europe, which were previously obtained by a conventional semi-nested PCR (**chapter VII**). The higher prevalence in analysed crayfish, especially in *P. leniusculus*, detected by the real-time PCR may have important implications for the management of these invaders.

- FUTURE PERSPECTIVES -

I hope that the present thesis contributes to our knowledge on invasive crayfish and crayfish plague and complements other lines of research, e.g., ecology of non-indigenous crayfish and the parasite, interactions with local organisms and their impact in Europe. My aim was also to inspire researchers to further study genetic variation in non-indigenous crayfish, especially some of the “New NICS”, in Europe, but also in their native ranges. The number of introduced crayfish species established in European waters continues to increase (e.g., Holdich et al. 2009, Jaklič and Vrezec 2011), representing a challenge for researchers to study these species and try to understand the process of their invasion.

The discovery of new lineages of *P. leniusculus* in North America (**chapter III**, E.R. Larson, pers. comm.) suggests that further studies should be conducted to clarify the

taxonomy of this crayfish. We are now cooperating with colleagues from Portugal (M. Lopes-Lima, Porto University) on the development of microsatellite markers for signal crayfish with subsequent analyses of its populations from Europe. These markers might allow the population structure of this crayfish to be studied or the history of its translocations among European countries to be documented; they could also be widely applied in its American range.

Results of DNA barcoding of non-indigenous North American crayfish in Europe (**chapter V**) already initiated at the Department of Ecology in Prague further activities focused on the identity and variation of recently established crayfish from the genus *Procambarus* in Europe. In *P. acutus* and *P.* cf. *zonangulus*, especially the taxonomy and the possibility of hybridization between them in a Dutch mixed population will be studied.

Some of the species that are invasive in Europe may be endangered in their native range. I had the opportunity to sample several populations of the spiny-cheek crayfish *Orconectes limosus* from its native area in Pennsylvania and Maryland where the species is endangered and I wish our publication (**chapter I**) attracts more attention to this crayfish in America. I hope it will be possible to examine genetic structure of endangered populations of these crayfish, e.g., using microsatellite markers (Hulák et al. 2010). Data on the processes which influence their genetic variation (e.g., genetic drift, connection or isolation of these populations, inbreeding) might then serve to determine Evolutionarily Significant Units (ESU) and help preserve them.

Detection of the crayfish plague in France (**chapter VI**) was a part of a three-year project. I had the opportunity to learn the TaqMan real-time PCR (Vrålstad et al. 2009) at the Norwegian Veterinary Institute in Oslo, Norway. I later transferred this technology to the laboratory of Ecology, Evolution, Symbiosis (EES) at the University of Poitiers, France where routine analyses of *A. astaci* presence in crayfish are now possible. We started with an analysis of more than 500 individuals of signal crayfish from numerous French populations (**chapter VI**). The project still continues and more samples of signal crayfish and other crayfish species from France are being analysed. When finished, the complete results will be provided to the French fishery organisation ONEMA. I hope our findings will contribute to increased efficiency in conservation efforts. Populations which are in imminent danger might be preferentially translocated to more suitable areas, e.g., to new isolated refuge sites known as “ark sites” (Peay 2009), and potential future eradication plans should target populations with the highest infection ratio (Oidtmann et al. 2006). Possible trends or patterns in the distribution of the disease in Europe could be uncovered when the crayfish plague prevalence in France is compared with results from other countries.

In the past, cultivation of the pathogen was required to identify different strains of *Aphanomyces astaci* and analyses of ribosomal ITS regions did not allow recognition of

basic groups (Makkonen et al. 2011). However, microsatellite markers for *A. astaci* are currently being developed in the Laboratory of Ecology, Evolution, Symbiosis (Poitiers, France) in collaboration with Trude Vrålstad (Norwegian Veterinary Institute in Oslo, Norway) and Javier Diéguez-Uribeondo (Royal Botanical Garden of Madrid, Spain). Once these markers are available, identification of strains found in invasive crayfish and those detected in dead native crayfish might show relationship between different hosts and strains of the pathogen, and reveal the real diversity of this pathogen in European waters. Easier discrimination between strains may also facilitate evaluation of their characteristics, such as their virulence, trace the origin of the pathogen when native crayfish mortalities appear, and possibly uncover the pathways of the spread of the parasite (e.g. Oidtmann et al. 2006, Vennerström et al. 1998). Data on the distribution of the crayfish plague in Europe are crucial for the efficient management of native crayfish and more such studies should be carried out, in a variety of crayfish invaders in European waters.

I believe that the cooperation I initiated between the Department of Ecology at Charles University in Prague (Czech Republic) and the Laboratory EES at the University of Poitiers (France) will continue in the future and that it will bring new interesting projects. In conclusion, I hope my work will stimulate further interest and more research projects focusing on crayfish and crayfish plague in both Europe and North America.

- 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.

Agerberg A. and Jansson H., 1995. Allozymic comparisons between three subspecies of the freshwater crayfish *Pacifastacus leniusculus* (Dana), and between populations introduced to Sweden. Hereditas, 122: 33-39.

Alderman D.J., 1996. Geographical spread of bacterial and fungal diseases of crustaceans. Revue Scientifique et Technique de l'Office International des Epizooties (Rev. Sci. Tech. Off. Int. Epiz.), 15: 603-632.

Alderman D.J., Polglase J.L. and Frayling M., 1987. *Aphanomyces astaci* pathogenicity under laboratory and field conditions. Journal of Fish Diseases, 10: 385-399.

Angeler D.G., Sánchez-Carrillo S., García G. and Alvarez-Cobelas M., 2001. The influence of *Procambarus clarkii* (Cambaridae, Decapoda) on water quality and sediment characteristics in a Spanish floodplain wetland. Hydrobiologia, 464: 89-98.

Aquiloni L., Martín M.P., Gherardi F. and Diéguez-Uribeondo J., 2011. The North American crayfish *Procambarus clarkii* is the carrier of the oomycete *Aphanomyces astaci* in Italy. Biological Invasions, 13: 359-367.

Arrignon J.C.V., Gépard P., Krier A. and Laurent P.J., 1999. The situation in Belgium, France, and Luxembourg. *In:* Crayfish in Europe as alien species. How to make the best of a bad situation? Gherardi F. and Holdich D.M. (Eds), 129-140. A.A. Balkema, Rotterdam, Brookfield.

Barbaresi S., Gherardi F., Mengoni A. and Souty-Grosset C., 2007. Genetics and invasion biology in fresh waters: a pilot study of *Procambarus clarkii* in Europe. *In:* Biological invaders in inland waters: profiles, distribution, and threats. Gherardi F. (Ed), 381-400. Invading Nature: Springer Series in Invasion Ecology. Springer, Dordrecht, The Netherlands.

Barbaresi S., Fani R., Gherardi F., Mengoni A. and Souty-Grosset C., 2003. Genetic variability in European populations of an invasive American crayfish: preliminary results. Biological Invasions, 5: 269-274.

Belfiore N.M. and May B., 2000. Variable microsatellite loci in red swamp crayfish, *Procambarus clarkii*, and their characterization in other crayfish taxa. Molecular Ecology, 9: 2155-2234.

Bickford D., Lohman D.J., Sodhi N.S., Ng P.K.L., Meier R., Winker K., Ingram K.K. and Das I., 2007. Cryptic species as a window on diversity and conservation. Trends in Ecology and Evolution, 22: 148-155.

Blanchet S., 2012. The use of molecular tools in invasion biology: an emphasis on freshwater ecosystems. Fisheries Management and Ecology, DOI 10.1111/j.1365-2400.2011.00832.x

Bondar C.A., Zhang Y., Richardson J.S. and Jesson D., 2005. The conservation status of the freshwater crayfish *Pacifastacus leniusculus*, in British Columbia. BC Ministry of Water, Land and Air Protection. Fisheries Management Report 117, pp 23.

Bouchard R.W., 1977. Distribution, systematic status and ecological notes on five poorly known species of crayfishes in western North America (Decapoda: Astacidae and Cambaridae). Freshwater Crayfish, 3: 409-423.

Bramard M., Demers A., Trouilhe M.-C., Bachelier E., Dumas J.-C., Fournier C., Broussard E., Robin O., Souty-Grosset C. and Grandjean F., 2006. Distribution of non-indigenous and non-indigenous crayfish populations in the Poitou-Charentes region (France): evolution over the past 25 years. Bulletin Français de la Pêche et de la Pisciculture, 380-381: 857-866.

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

Busack C.A., 1988. Electrophoretic variation in the red swamp (*Procambarus clarkii*) and white river crayfish (*P. acutus*) (Decapoda: Cambaridae). Aquaculture, 69: 211-226.

Cammà C., Ferri N., Zezza D., Marcacci M., Paolini A., Ricchiuti L. and Lelli R., 2010. Confirmation of crayfish plague in Italy: detection of *Aphanomyces astaci* in white clawed crayfish. Diseases of Aquatic Organisms, 89: 265-268.

Cao L., Zhou L. and Zhang B. 2010. Genetic patterns of an invasive *Procambarus clarkii* population in the three river basins of Anhui Province. Biodiversity Science, 18: 398-407. (in Chinese with English abstract)

Cerenius L. and Söderhäll K., 1984. Repeated zoospore emergence from isolated spore cysts of *Aphanomyces astaci*. Experimental Mycology, 8: 370-377.

Cerenius L., Söderhäll K., Persson M. and Ajaxon R., 1988. The crayfish plague fungus *Aphanomyces astaci* - diagnosis, isolation, and pathobiology. Freshwater Crayfish, 7: 131-144.

Collas M., Julien C. and Monnier D., 2007. Note technique. La situation des écrevisses en France. Résultats des enquêtes nationales réalisées entre 1977 et 2006 par le Conseil Supérieur de la pêche [Technical note. Situation of the crayfish in France. Results of the national surveys performed between 1977 and 2006 by the Conseil Supérieur de la pêche (CSP)]. Bulletin Français de la Pêche et de la Pisciculture, 386: 01-38.

Collas M. and Salek X., 2002. Description d'un cas de peste ou *Aphanomycose* dans le département des Vosges [Description of an outbreak of crayfish plague or aphanomycosis in Vosges department]. L'Astaciculteur de France, 70: 2-6.

Cunningham A.A., Daszak P. and Rodríguez J.P., 2003. Pathogen pollution: defining a parasitological threat to biodiversity conservation. The Journal of Parasitology, 89: S78-S83.

DAISIE European Invasive Alien Species Gateway, 2008. 100 of the worst. Available from http://www.europe-aliens.org/speciesTheWorst.do [accessed on December 2, 2011]

Daszak P., Cunningham A.A. and Hyatt A.D., 2003. Infectious disease and amphibian population declines. Diversity and Distributions, 9: 141-150.

Diéguez-Uribeondo J., Cerenius L., Dyková I., Gelder S., Henttonen P., Jiravanichpaisal P., Lom J. and Söderhäll K., 2006. Pathogens, parasites and ectocommensals. *In:* Atlas of crayfish in Europe. Souty-Grosset C., Holdich D.M., Noël P.Y., Reynolds J.D. and Haffner P. (Eds), 133-149. Patrimoines naturels, 64. Muséum national d'Histoire naturelle, Paris.

Diéguez-Uribeondo J., Huang T.S., Cerenius L. and Söderhäll K., 1995. Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii*. Mycological Research, 99: 574-578.

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

Edgerton B.F., Henttonen P., Jussila J., Mannonen A., Paasonen P., Taugbøl T., Edsman L. and Souty-Grosset C., 2004. Understanding the causes of disease in European freshwater crayfish. Conservation Biology, 18: 1466-1474.

EEA, 2007. Halting the loss of biodiversity by 2010: proposal for a first set of indicators to monitor progress in Europe. European Environment Agency, technical report 11/2007, pp 182.

Estoup A. and Guillemaud T., 2010. Reconstructing routes of invasion using genetic data: why, how and so what? Molecular Ecology, 19: 4113-4130.

Fitzpartick B.M., Fordyce J.A., Niemiller M.L. and Reynolds R.G., 2011. What can DNA tell us about biological invasions? Biological Invasions, 14: 245-253.

Gherardi F., 2007. Biological invasions in inland waters: an overview. *In:* Biological invaders in inland waters: profiles, distribution and threats. Gherardi F. (Ed), 3-25. Springer, Dordrecht, The Netherlands.

Gozlan R.E., Whipps C.M., Andreou D. and Arkush K.D., 2009. Identification of a rosette-like agent as *Sphaerothecum destruens*, a multi-host fish pathogen. International Journal for Parasitology, 39: 1055-1058.

Grandjean F. and Souty-Grosset C., 1997. Preliminary results on the genetic variability of mitochondrial DNA in the signal crayfish, *Pacifastacus leniusculus* Dana. Life Sciences, 320: 551-556.

Harlioğlu A.G. and Harlioğlu M.M., 2009. The status of freshwater crayfish (*Astacus leptodactylus* Eschscholtz) fisheries in Turkey. Reviews in Fisheries Science, 17: 187-189.

Hänfling B., Edwards F. and Gherardi F., 2011. Invasive alien Crustacea: dispersal, establishment, impact and control. BioControl, 56: 573-595.

Henttonen P. and Huner J.V., 1999. The introduction of alien species of crayfish in Europe: A historical introduction. *In:* Crayfish in Europe as alien species. How to make the best of a bad situation? Gherardi F. and Holdich D.M. (Eds), 13-22. A.A. Balkema, Rotterdam, Brookfield.

Hochwimmer G., Tober R., Bibars-Reiter R., Licek E. and Steinborn R., 2009. Identification of two GH18 chitinase family genes and their use as targents for detection of the crayfish-plague oomycete *Aphanomyces astaci*. BMC Microbiology, 9: 184.

Holdich D.M., 1999. The negative effects of established crayfish introductions. *In:* Crayfish in Europe as alien species. How to make the best of a bad situation? Gherardi F. and Holdich D.M. (Eds), 31-47. A.A. Balkema, Rotterdam, Brookfield.

Holdich D.M., 2003. Crayfish in Europe – an overview of taxonomy, legislation, distribution, and crayfish plague outbreaks. *In:* Management and Conservation of Crayfish. Proceedings of a conference held on 7th November 2002 in Nottingham, UK. Holdich D.M. and Sibley P. J. (Eds), 15-34. Environment Agency, Bristol, UK.

Holdich D.M. and Pöckl M., 2007. Invasive crustaceans in European inland waters. *In:* Biological invaders in inland waters: Profiles, distribution, and threats. Gherardi F. (Ed), 29-75. Springer, Dordrecht, The Netherlands.

Holdich D.M., Reynolds J.D., Souty-Grosset C. and 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, 394-395: art. no. 11.

Hulák M., Kašpar V., Kozák P., Buřič M., Filipová L. and Petrusek A., 2010. Cross-species amplification of microsatellite markers in the invasive spiny-cheek crayfish (*Orconectes limosus*): assessment and application. Journal of Applied Genetics, 51: 73-78.

Hulme P.E., Bacher S., Kenis M., Klotz S., Kühn I., Minchin D., Nentwig W., Olenin S., Panov V., Pergl J., Pyšek P., Roques A., Sol D., Solarz W. and Vilà M., 2008. Grasping at the routes of biological invasions: a framework for integrating pathways into policy. Journal of Applied Ecology, 45: 403-414.

Jaklič M. and Vrezec A., 2011. The first tropical alien crayfish species in European waters: the redclaw *Cherax quadricarinatus* (von Martens, 1868) (Decapoda, Parastacidae). Crustaceana, 84: 651-665.

Jeschke J.M. and Strayer D.L., 2005. Invasion success of vertebrates in Europe and North America. PNAS, 102: 7198-7202.

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.

Kolbe J.J., Glor R.E., Rodríguez Schettino L., Lara A.C., Larson A. and Losos J.B., 2004. Genetic variation increases during biological invasion by a Cuban lizard. Nature, 431: 177-181.

Kossakowski J., 1966. Raki. Panstwowe wydawnictwo rolnicke i lesne, Warszawa, Poland.

Kozubíková E., Petrusek A., Ďuriš Z., Martín M.P., Diéguez-Uribeondo J. and 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 M.P., Diéguez-Uribeondo J., Oidtmann B. and 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. and Petrusek A., 2010. Crayfish plague pathogen in invasive North American crayfish species in Hungary. Journal of Fish Diseases, 33: 925-929.

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

Larson E.R. and Olden J.D., 2011. The state of crayfish in the Pacific Northwest. Fisheries, 36: 60-73.

Lieb D.A., Bouchard R.W., Carline R.F., Nuttall T.R., Wallace J.R. and Burkholder C.L., 2011. Conservation and management of crayfishes: lessons from Pennsylvania. Fisheries, 36: 489-507.

Longshaw M., 2011. Diseases of crayfish: A review. Journal of Invertebrate Pathology. 106: 54-70.

Lowe S.J., Browne M. and Boudjelas S., 2000. 100 of the World's Worst Invasive Alien Species. IUCN/SSC Invasive Species Specialist Group (ISSG), Auckland, New Zealand, pp 12.

Mack R.N., Chair, Simberloff D., Lonsdale W.M., Evans H., Clout M. and Bazzaz F., 2000. Biotic invasions: causes, epidemiology, global consequences and control. Issues in Ecology, 5: 1-20.

Makkonen J., Jussila J., Henttonen P. and Kokko H., 2011. Genetic variation in the ribosomal internal transcribed spacers of *Aphanomyces astaci* Schikora from Finland. Aquaculture, 311: 48-53.

Mathews L.M., Adams L., Anderson E., Basile M., Gottardi E. and Buckholt M.A., 2008. Genetic and morphological evidence for substantial hidden biodiversity in a freshwater crayfish species complex. Molecular Phylogenetics and Evolution, 48: 126-135.

Mathews L.M. and Warren A.H., 2008. A new crayfish of the genus *Orconectes* Cope, 1872 from southern New England (Crustacea: Decapoda: Cambariidae). Proceedings of the Biological Society of Washington, 121: 374-381.

Mergeay J., Verschuren D. and De Meester L., 2006. Invasion of an asexual American water flea clone throughout Africa and rapid displacement of a native sibling species. Proceedings of the Royal Society London B Biological Sciences, 273: 2839-2844.

Miller G.C., 1960. The taxonomy and certain biological aspects of the crayfish of Oregon and Washington. Master's thesis, Oregon State College, Corvalis, pp 216.

Neveu A., 2002. *Pacifastacus leniusculus*. In: Les espèces animales et végétales susceptibles de proliférer dans les milieux aquatiques et subaquatiques. Fiches - espèces animales [Animal and plant species capable to proliferate in aquatic and submerged areas. Files – animal species]. Rapport de DESS, Agence de l'Eau Artois-Picardie (Douai), 87-91.

Oidtmann B., Bausewein S., Hölzle L., Hoffmann R. and Wittenbrink M., 2002. Identification of the crayfish plague fungus *Aphanomyces astaci* by polymerase chain reaction and restriction enzyme analysis. Veterinary Microbiology, 85: 183-194.

Oidtmann B., Cerenius L., Schmid I., Hoffmann R. and Söderhäll K., 1999. Crayfish plague epizootics in Germany - classification of two German isolates of the crayfish plague fungus *Aphanomyces astaci* by random amplification of polymorphic DNA. Diseases of Aquatic Organisms, 35: 235-238.

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

Oidtmann B., Heitz E., Rogers D. and Hoffmann R.W., 2002. Transmission of crayfish plague. Diseases of Aquatic Organisms, 52: 159-167.

Pârvulescu L., Schrimpf A., Kozubíková E., Resino S.C., Vrålstad T., Petrusek A. and 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*, in press*. DOI 10.3354/dao02432

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

Peeler E.J. and Feist S.W., 2011. Human intervention in freshwater ecosystems drives disease emergence. Freshwater Biology, 56: 705-716.

Plowright W., 1988. Research on wildlife diseases: is a reappraisal necessary? Revue Scientifique et Technique de l'Office International des Epizooties (Rev. Sci. Off. Int. Epiz.), 7: 783-795.

Poulin R., Paterson R.A., Townsend C.R., Tompkins D.M. and Kelly D.W., 2010. Biological invasions and the dynamics of endemic diseases in freshwater ecosystems. Freshwater Biology, 56: 676-688.

Ricciardi A., Palmer M.E. and Yan N.D., 2011. Should biological invasions be managed as natural disasters? BioScience, 61: 312-317.

Roman J. and Darling J.A., 2007. Paradox lost: genetic diversity and the success of aquatic invasions. Trends in Ecology and Evolution, 22: 454-464.

Sakai A.K., Allendorf F.W., Holt J.S., Lodge D.M., Molofsky J., With K.A., Baughman S., Cabin R.J., Cohen J.E., Ellstrand N.C., McCauley D.E., O'Neil P., Parker I.M., Thompson J.N. and Weller S.G., 2001. The population biology of invasive species. Annual Review of Ecology and Systematics, 32: 305-332.

Scalici M., Chiesa S., Gherardi F., Ruffini M., Gibertini G. and Marzano F.N., 2009. The new threat to Italian inland waters from the alien crayfish "gang": the Australian *Cherax destructor* Clark, 1936. Hydrobiologia, 632: 341-345.

Schikora F., 1916. Die Wiederbevölkerung der deutschen Gewässer mit Krebsen. Emil Hübner Verlag, Bautzen, Germany.

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. Biological Invasions, 13: 1269-1274.

Sonntag M.M., 2006. Taxonomic standing of the three subspecies of *Pacifastacus leniusculus*, and their phylogeographic patterns in the Klamath Basin area. Brigham Young University, pp 71.

Souty-Grosset C., Holdich D.M., Noël P.Y., Reynolds J.D. and Haffner P., 2006. Atlas of crayfish in Europe. Patrimoines naturels, 64. Muséum national d'Histoire naturelle, Paris.

Spickler A.R., 2010. Infectious Salmon Anemia. The Center for Food Security and Public Health. Available from http://www.cfsph.iastate.edu/Factsheets/pdfs/infectious_salmon_anemia.pdf [accessed on February 5, 2012]

Strand D.A., Holst-Jensen A., Viljugrein H., Edvardsen B., Klaveness D., Jussila J. and 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.

Strayer D.L., 2010. Alien species in fresh waters: ecological effects, interactions with other stressors, and prospects for the future. Freshwater Biology, 55: 152-174.

Svoboda J., Kozubíková E., Kozák P., Kouba A., Bahadir Koca S., Diler Ö., Diler I., Policar T. and Petrusek A., 2012. PCR detection of the crayfish plague pathogen in narrow-clawed crayfish inhabiting Lake Eğirdir in Turkey. Diseases of Aquatic Organisms, *in press*. DOI 10.3354/dao02445

Swecker C.D., Jones T.G., Donahue K. II, Mckinney D. and Smith G.D., 2010. The extirpation of *Orconectes limosus* (spinycheek crayfish) populations in West Virginia. Southeastern Naturalist, 9(special issue 3): 155-164.

Torchin M.E., Lafferty K.D., Dobson A.P., McKenzie V.J. and Kuris A.M., 2003. Introduced species and their missing parasites. Nature, 421: 628-630.

Torrez E. and Álvarez F., 2011. Genetic variation in native and introduced populations of the red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Crustacea, Decapoda, Cambaridae) in Mexico and Costa Rica. Aquatic Invasions, *in press*. DOI 10.3391/ai.2011.accepted

Tuffs S. and 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., 1973. Significance of diseases on freshwater crayfish. Freshwater Crayfish, 1: 135-150.

Unestam T., 1975. Defence reactions in and susceptibility of Australian and New Guinean freshwater crayfish to European-crayfish-plague fungus. Australian Journal of Experimental Biology and Medical Science, 53: 349-359.

Vennerström P., Söderhäll K. and Cerenius L., 1998. The origin of two crayfish plague (*Aphanomyces astaci*) epizootics in Finland on noble crayfish, *Astacus astacus*. Annales Zoologici Fennici, 35: 43-46.

Vilà M., Basnou C., Pyšek P., Josefsson M., Genovesi P., Gollasch S., Nentwig W., Olenin O., Roques A., Roy D. et al., 2010. How well do we understand the impacts of alien species on ecosystem services? A pan-European, cross-taxa assessment. Frontiers in Ecology and the Environment, 8: 135-144.

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. Bulletin of the European Association of Fish Pathologists, 31: 182-188.

Vrålstad T., Johnsen S.I., Fristad R.F., Edsman L. and 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. and 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.

Weijden W. van der, Leewis R. and Bol P., 2007. Biological globalisation: Bio-invasions and their impacts on nature, the economy and public health. KNNV Publishing, Utrecht, The Netherlands, 224 p.

Westman K., 2002. Alien crayfish in Europe: negative and positive impacts and interactions with native crayfish. *In:* Invasive aquatic species of Europe: distribution, impacts, and management. Leppäkoski E., Gollash S. and Olenin S. (Eds), 76-95. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Williamson M., 1996. Biological invasions. London: Chapman and Hall, pp 244.

Yue G.H., Wang G.L., Zhu B.Q., Wang C.M., Zhu Z.Y. and Lo L.C., 2008. Discovery of four natural clones in a crayfish species *Procambarus clarkii*. International Journal of Biological Sciences, 4: 279-282.

Yue G. H., Li J., Bai Z., Wand C. M. and Feng F., 2010. Genetic diversity and population structure of the invasive alien red swamp crayfish. Biological Invasions, 12: 2697-2706.

Zhu Z.Y. and Yue G.H., 2008. Eleven polymorphic microsatellites isolated from red swamp crayfish, *Procambarus clarkii*. Molecular Ecology Resources, 8: 796-798.