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

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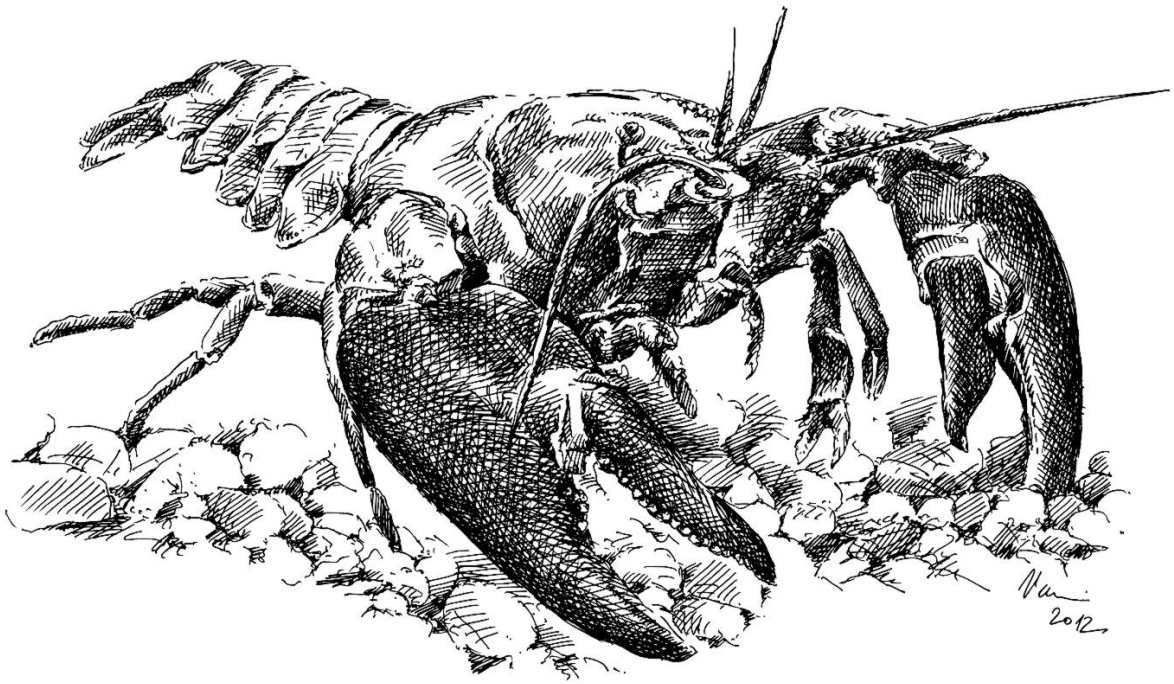
Prague, February 2012

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



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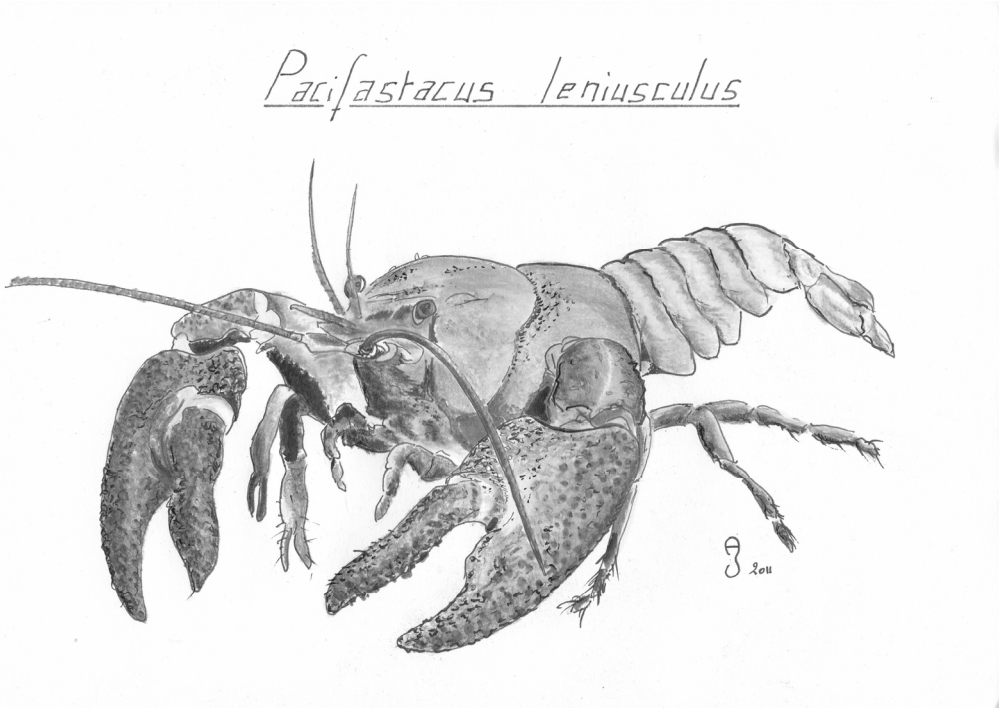

- PREFACE AND ACKNOWLEDGEMENTS -

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.

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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

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- 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) -

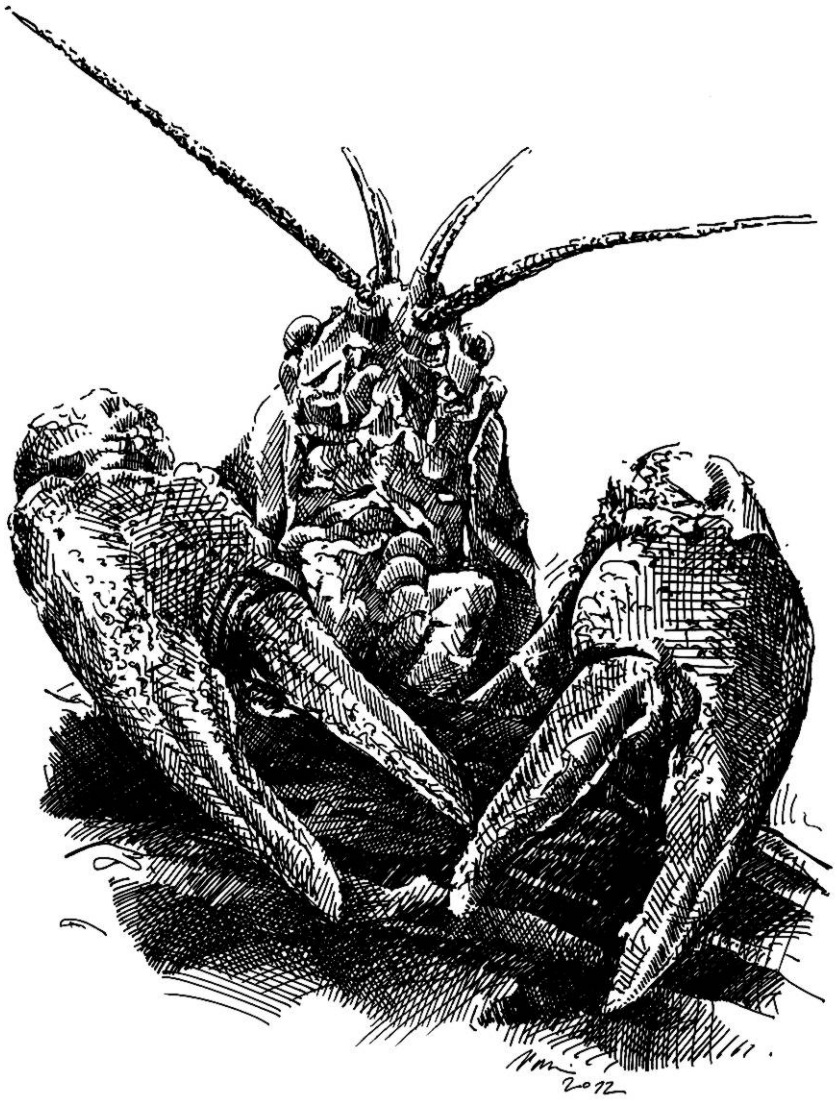
Les invasions biologiques de crustacés représentent une sérieuse menace pour les espèces natives d'Europe. Dans ma thèse je me suis intéressée aux écrevisses non-indigènes introduites en Europe et à leur parasite *Aphanomyces astaci*, le pathogène de la peste de l'écrevisse. La thèse est composée de 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 II**). 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.

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- PUBLICATIONS AND MANUSCRIPTS -

PART 1

Genetic variation in crayfish invaders

CHAPTER I

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Haplotype variation in the spiny-cheek crayfish *Orconectes limosus*: colonization of Europe and genetic diversity of native stocks

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Abstract. The North American spiny-cheek crayfish, *Orconectes limosus* (Rafinesque, 1817), a widespread invader in Europe, seems to have been introduced there successfully only once. According to available literature, 90 individuals of unclear origin were released in Poland in 1890. Despite this apparent bottleneck, the species has successfully colonized various aquatic habitats and has displaced native crayfish species in many places. To test whether different European populations were likely to have come from a single source and to identify their possible origin, we analyzed the diversity of the mitochondrial gene for cytochrome c oxidase subunit I (COI) of *O. limosus* individuals from Europe and from its original range in North America, including the presumed source region of European populations, the Delaware River watershed (eastern USA). Two haplotypes were found in European populations. One haplotype was widespread; the other was present in a single population. In contrast, 18 haplotypes were detected in North America. This result supports the hypothesis of a single overseas introduction of *O. limosus* and suggests that the high invasion success of this species was not limited by an introduction bottleneck. Two divergent clades were detected in North American *O. limosus* populations. One, which includes the dominant haplotype in Europe, was found in a large part of the species' present range. The 2nd (diverging by >1%) was mostly restricted to a limited area in southeastern Pennsylvania. *Orconectes limosus* populations in the northern part of the species' North American range, at least some of which are nonindigenous themselves, may share the source area with European *O. limosus*. The endangered status of *O. limosus* populations in southeastern Pennsylvania and northeastern Maryland, where much of the species' genetic diversity resides, should be considered in conservation management.

Key words: invasive crayfish, Cambaridae, *Orconectes limosus*, COI variation, Europe, introduction history, conservation.

Several nonindigenous crayfish species, most from North America, have been introduced to Europe since the 19th century to replace lost populations of native species decimated by crayfish plague, which was accidentally introduced to Europe in 1859 (Souty-Grosset et al. 2006). Introduced crayfish in Europe can be economically beneficial, but they have negative effects on local environments. In particular, they directly endanger native crayfish. Apart from interspecific competition, North American crayfishes transmit the crayfish plague pathogen, the oomycete *Aphanomyces astaci* Schikora, 1903, to indigenous species. This infection causes mass mortalities and further reduces native crayfish numbers in areas invaded by North American species (Holdich 1999). Introduction of the crayfish plague pathogen apparently preceded colonization of European waters by North American crayfish, the first of which were intentionally imported in the 1890s. Therefore, the original vector of the crayfish plague remains unknown.

The 3 most widespread invasive American crayfishes in Europe differ in their colonization history.

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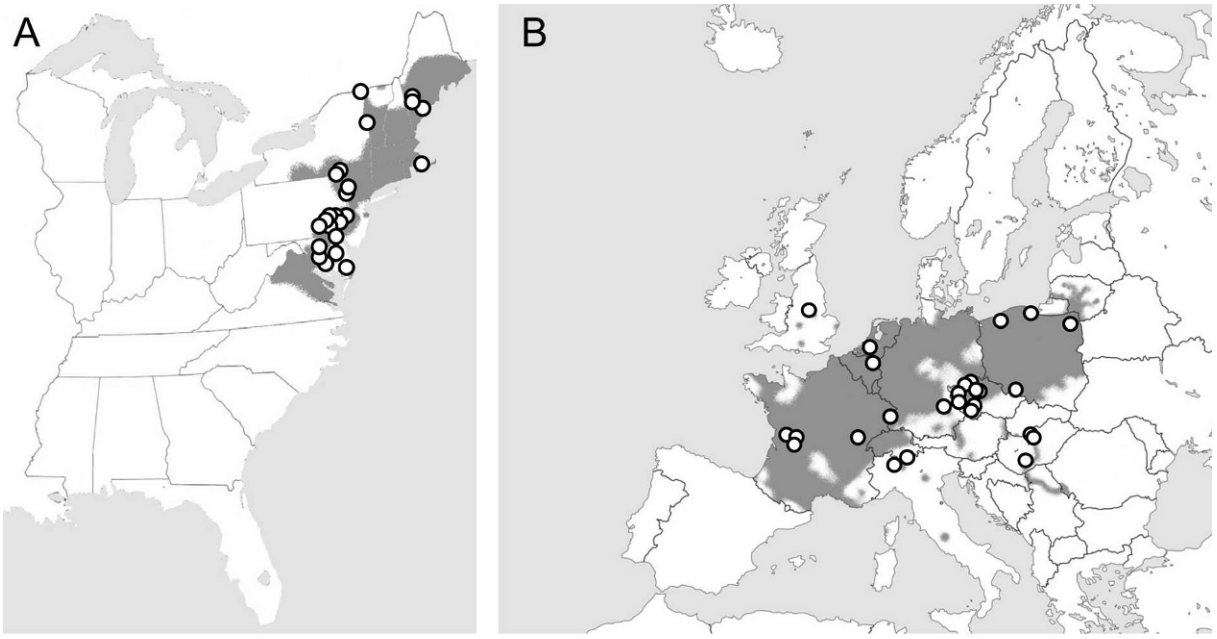

FIG. 1. The current distribution of *Orconectes limosus* in the USA (A) and Europe (B). Shaded areas show the distribution of the species. Dots show approximate source localities of individuals included in our study (symbols of adjacent localities may overlap). The US distribution was compiled from Aiken (1965), Bouchard et al. (2007), Crocker (1957, 1979), Egnotovich (2006), Fetzner (1999–2006), Francois (1959), Gelder et al. (2001), Hobbs (1972, 1989), Jezerinac et al. (1995), Kazyak et al. (2005), Kilian et al. (2010), Kuhlmann and Hazelton (2007), Lieb et al. (2011a, b), Loughman et al. (2009), Ortmann (1906), Rhoades (1962), Vaux (2009), and L. Matthews (Vermont Agency of Natural Resources, personal communication). The single population from Nova Scotia, Canada, is not shown because of its distance from other North American populations. The European range was based on Janský and Kautman (2007), Pârvulescu et al. (2009), Pavlovič et al. (2006), and Souty-Grosset et al. (2006).

Two of them, the signal crayfish *Pacifastacus leniusculus* (Dana, 1852), and the red swamp crayfish *Procambarus clarkii* (Girard, 1852), were brought to Europe several times and in large numbers. More than 100,000 *P. leniusculus* were introduced in the 1960s and ~40,000 *P. clarkii* were introduced in 1973 (Henttonen and Huner 1999, Skurdal et al. 1999, Souty-Grosset et al. 2006).

Introduction of the 3rd widespread crayfish invader, the spiny-cheek crayfish *Orconectes limosus*, preceded the others by almost 60 y. It was first brought to Europe in 1890, when 90 individuals of a batch sent by the US Commission of Fish and Fisheries survived overseas transport (McDonald 1893) and were released to a fishpond near Barnówko (Berneuchen) in Pomerania, currently in western Poland (Kossakowski 1966). This release seems to be the only known successful introduction of *O. limosus* to Europe (Kulmatycki 1935). Another recorded attempt to introduce it from New York to France in 1895 failed (Kossakowski 1966).

The exact origin of European *O. limosus* is unknown. The North American range of the species is on the eastern coast of the USA (Fig. 1A) and Canada, but its present distribution has been affected by human activities (Souty-Grosset et al. 2006, Lambert et al. 2007). Authors of several publications, such as Kossakowski (1966), Henttonen and Huner (1999), and Holdich (2003), claimed that the *O. limosus* introduced to Europe came from the Delaware River in the northeastern USA. However, this statement, taken from Schikora (1916), does not seem to be based on any reliable data. The original report on the species' overseas transport (McDonald 1893) does not provide any information, so more recent publications (Souty-Grosset et al. 2006, Holdich and Black 2007) leave the question about the true origin of the European stock open.

During the century after its introduction to Europe, *O. limosus* spread rapidly to various regions, both naturally and by secondary human-mediated introductions (Souty-Grosset et al. 2006). Currently, it can be found in $\geq$20 European countries, and it has recently invaded, via the Danube River, Slovakia (Janský and Kautman 2007) and Romania (Pârvulescu et al. 2009). In addition, it has been introduced to Morocco in North Africa (Huner 1988). The present distribution of the species in Europe and the USA is summarized in Fig. 1A, B. In Europe, *O. limosus* is

considered an invasive pest, but it is threatened by other aggressively spreading species of cambarid crayfish in some parts of its original North American range (Bouchard et al. 2007, Kilian et al. 2010, Lieb et al. 2011a, b).

The successful spread of *O. limosus* in Europe can be explained by its ecological plasticity, tolerance to poor environmental conditions, and reduced competition with native crayfish populations via transmission of crayfish plague (Lindqvist and Huner 1999, Kozubíková et al. 2009). In addition, under certain conditions, *O. limosus* is capable of facultative parthenogenetic reproduction (Buřič et al. 2011), which might contribute to its invasive success. The disjunct distribution of *O. limosus* in Europe can be explained by a combination of natural dispersal from the region of 1st introduction via rivers and canals and long-range transport by humans. However, an alternative scenario, that undocumented introduction(s) from the original distribution area increased the colonized range in Europe and the species' genetic diversity, cannot be completely disregarded. Our preliminary analyses of intrapopulation genetic variation of several central European populations based on 8 allozyme loci (Filipová et al. 2009) and 10 microsatellite loci (Hulák et al. 2010) suggested that *O. limosus* populations are diverse even within the small area for which the introduction scenario is supposed to be relatively simple (Petrusek et al. 2006). Substantial variation at nuclear loci may have been retained from the original population. However, we presumed that the introduction bottleneck documented in the literature, if true, would have had a much more profound effect on variation in the species' mitochondrial deoxyribonucleic acid (mtDNA) in Europe.

The aims of our study were to test whether all European populations of *O. limosus* come from a single source, and, if possible, to identify the source area within the species' native range. Authors of other studies on cambarid crayfish showed substantial divergences of mitochondrial lineages among various geographic regions or watersheds in their native ranges (Fetzner and Crandall 2003, Mathews et al. 2008). We hypothesized that *O. limosus* would show similar patterns. If so, the scenarios of a single introduction of the species to Europe vs multiple introductions from various sources would predict different levels of variation of mtDNA markers (low and high, respectively) in the invaded range. To test these hypotheses, we compared sequences of the cytochrome c oxidase subunit I (COI) gene fragment of randomly selected individuals from different European populations of *O. limosus* with those from its North American range, including the supposed source region for the 1890 introduction. Unfortunately, the number of available specimens from most populations was low, so we were unable to evaluate geographic patterns of more-variable nuclear markers.

Methods

We analyzed the diversity of a 639-basepair (bp) fragment of the mitochondrial COI gene from 297 *O. limosus* individuals representing 70 localities (Table 1). We made an effort to collect *O. limosus* from representative localities in its native and introduced ranges in North America (for the status of populations see Table 1) and in its invaded European range. The samples came from Maine, Maryland, Massachusetts, Pennsylvania, Vermont, and Nova Scotia in North America (206 individuals from 44 populations; Figs 1A, 2A–C) and from Belgium, Czech Republic, France, Germany, Great Britain, Hungary, Italy, The Netherlands, and Poland in Europe (91 individuals from 25 populations; Fig. 1B). We included in the analysis samples from the Delaware River watershed (Pennsylvania and New York, USA), a potential source region of animals introduced to Europe in 1890. In Europe, we included more localities from the Czech Republic to cover populations and individuals showing differences in allozyme markers (Filipová et al. 2009), to maximize the chance that divergent mtDNA haplotypes, if present, would be detected. We also used 3 sequences available in GenBank, 2 from Poland (accession numbers AF517105, DQ882096) and 1 from New York (AY701199), in the analysis.

We isolated total genomic DNA from the muscle tissue of 1 leg segment of each captured crayfish with a Chelex® extraction protocol. We homogenized ~1 mm^3 of the tissue in a solution of 175 μL of distilled water and 5 μL of proteinase K (20 mg/mL) (Promega, Madison, Wisconsin) and homogenized the content again after adding another 175 μL of distilled water. Next, we added ~50 μL of Chelex 100 beads (Sigma–Aldrich, St Louis, Missouri), vortexed the Eppendorf tubes gently, and incubated them at 56°C for 4 h, followed by incubation at 100°C for 8 min to denature proteins. Subsequent centrifugation for 4 min at 12,000 rpm ensured removal of the Chelex resin and undigested solids from the suspension. We stored the resulting supernatant at −20°C.

We carried out the amplifications of the COI fragment in 25 μL volumes with HCO 2198 and LCO 1490 primers (Folmer et al. 1994). The polymerase chain reaction (PCR) mixture contained a PCR buffer (1.5 mM $MgCl_2$) (Promega), 200 μM of deoxynucleotide triphosphates (dNTPs), 25 μM of

TABLE 1. Localities sampled for *Orconectes limosus* in North America and Europe, with the number of individuals analyzed (*n*) and haplotypes detected. Numbers in parentheses indicate the number of individuals with that particular haplotype. If no number is provided, all individuals from the respective population carried the same haplotype. Countries, US states, and Canadian provinces are indicated by 2-letter abbreviations (Canada: NS = Nova Scotia; USA: MA = Massachusetts, MD = Maryland, ME = Maine, NY = New York, PA = Pennsylvania, VT = Vermont; Europe: BE = Belgium, CZ = Czech Republic, DE = Germany, FR = France, GB = United Kingdom, HU = Hungary, IT = Italy, NL = Netherlands, PL = Poland). Watersheds in the USA are distinguished as upper Delaware River Basin (UD), lower Delaware River Basin (LD), Schuylkill River Basin (SK), Susquehanna River Basin (S), and Chesapeake Bay Basin (CB). Asterisks indicate localities analyzed in previous studies, with sequences deposited in GenBank. Status of North American populations is native (N), introduced (I), probably introduced (PI), and uncertain (U). Status assessments for these populations were based on the following sources: Nova Scotia and New England (Crocker 1979, Smith 1997, Lambert et al. 2007), UD (Bouchard et al. 2007), LD (Ortmann 1906, Bouchard et al. 2007), S (assessment of DAL), and MD (Meredith and Schwartz 1960, Kilian et al. 2010). Trib. = tributary.

Political unit (watershed)	Locality	County/region	Sampling date	Latitude	Longitude	Haplotype codes	*n*	Status
North America								
Canada								
NS	Freshwater Lake, Ingonish Beach	Cape Breton	Aug 2009	46°38′N	60°24′W	A1(3), A3(6)	9	I
USA								
ME	Horseshoe Pond	Oxford	1 Sep 2009	44°13′N	70°56′W	A1	1	I
ME	Kezar Lake	Oxford	1 Sep 2009	44°09′N	70°56′W	A1	4	I
ME	Pleasant River	Oxford	Nov 2009	43°46′N	70°26′W	A1	2	I
ME	Stroundwater River	Cumberland	28 Jul 2003	43°40′N	70°22′W	A1	9	I
VT	Lake Champlain, Grand Isle	Grand Isle	5 Aug 2003	44°43′N	73°20′W	A1	1	I
VT	Lewis Creek, Ferrisburgh	Addison	20 Oct 1993	44°15′N	73°14′W	A5	1	I
MA	Mashpee River	Barnstable	27 Oct 2009	41°39′N	70°29′W	A13	10	I
NY (UD)	East Branch Delaware River*	Delaware	1 Oct 2002	41°58′N	75°11′W	B1	1	PI
PA (UD)	West Branch Delaware River	Wayne	Jun 2005, Oct 2009	41°57′N	75°17′W	B1	2	PI
PA (UD)	Upper Delaware River	Wayne	29 Sep 2009	41°52′N	75°16′W	A1(6), B1(3)	9	PI
PA (UD)	Calkins Creek	Wayne	6 Jun 2005	41°40′N	75°04′W	B1	3	PI
PA (UD)	Raymondskill Creek	Pike	7 Jun 2005	41°17′N	74°50′W	A1(5), B1(1)	6	PI
PA (UD)	Dingmans Creek	Pike	8 Jun 2005	41°14′N	74°54′W	A1	5	PI
PA (UD)	Hornbecks Creek	Pike	9 Jun 2005	41°12′N	74°55′W	A1	10	PI
PA (UD)	Brodhead Creek	Monroe	13 Jul 2005	41°00′N	75°08′W	B4	1	PI
PA (LD, SK)	Manatawny Creek	Berks	25 May 2006	40°19′N	75°44′W	A2(1), B1(5)	6	U
PA (LD, SK)	Stony Run	Chester	25 May 2006	40°10′N	75°35′W	B1	3	U
PA (LD)	Valley Creek	Chester	May 2006, Aug 2009	39°59′N	75°40′W	B1	9	N
PA (LD)	Neshaminy Creek	Bucks	21 Sep 2009	40°08′N	74°55′W	A3(4), B1(1), B5(3)	8	N
PA (LD)	Lower Delaware River	Bucks	28 Aug 2009	40°06′N	74°51′W	A3	2	N
PA (LD)	Lower Delaware River	Philadelphia	29 Aug 2009	40°03′N	74°59′W	A3(1), A12(1)	2	N
PA (LD)	Pennypack Creek	Philadelphia	21 Sep 2009	40°04′N	75°03′W	B1(4), B5(3)	7	N
PA (LD)	Crum Creek	Delaware	May 2006, Nov 2008	39°59′N	75°26′W	B1	2	N
PA (LD)	Ridley Creek	Delaware	11 May 2006	39°57′N	75°27′W	B1(4), B2(2)	6	N
PA (LD)	West Branch Chester Creek	Delaware	11 May 2006	39°53′N	75°30′W	B1(5), B3(1)	6	N
PA (LD)	Brandywine Creek	Delaware	10 May 2006	39°52′N	75°36′W	A3(1), B1(5)	6	N
PA (LD)	Buck Run	Chester	10 May 2006	39°56′N	75°50′W	B1	6	N
PA (LD)	East Branch White Clay Creek	Chester	12 May 2006	39°52′N	75°47′W	B1	6	N
PA (S)	East Branch Octoraro Creek	Lancaster/Chester	20 Sep 2009	39°54′N	76°00′W	A9(3), A10(5)	8	U
PA (S)	Valley Run	Lancaster	19 Sep 2009	39°56′N	76°03′W	A10	1	U
PA (S)	Meetinghouse Creek	Lancaster	19 Sep 2009	39°55′N	76°05′W	A9	10	U

TABLE 1. Continued.

Political unit (watershed)	Locality	County/region	Sampling date	Latitude	Longitude	Haplotype codes	*n*	Status
PA (C)	Big Elk Creek	Chester	11 May 2006	39°44′N	75°51′W	A3	1	N
MD (C)	Big Elk Creek	Cecil	10 Jun 2008	39°39′N	75°49′W	A3(2), A6(2), A11(1)	5	N
MD (C)	Little Elk Creek	Cecil	3 Jun 2008	39°42′N	75°53′W	A3	2	N
MD (C)	Gravelly Run	Cecil	3 Jun 2008	39°38′N	75°52′W	A3	5	N
MD (C)	Yellow Branch	Harford	30 Jun 2008	39°34′N	76°31′W	A7	5	N
MD (C)	Little Gunpowder Falls	Baltimore/ Harford	22 Jun 2007	39°31′N	76°30′W	A7	2	N
MD (C)	Deep Run	Howard	12 Jun 2007	39°11′N	76°43′W	A1	3	N
MD (C)	Unnamed trib. to Southwest Branch	Prince Georges's	10 Jul 2008	38°54′N	76°51′W	A2	1	N
MD (C)	Clark Run	Charles	12 Aug 2008	38°31′N	76°58′W	A1	1	N
MD (C)	Ross Branch	Charles	13 Aug 2008	38°27′N	76°58′W	A1	1	N
MD (C)	Unnamed trib. to St. Clements Creek	St. Mary's	21 Jun 2007	38°22′N	76°44′W	A1	2	N
MD (C)	Unnamed trib. to Beaverdam Ditch	Queen Anne's	16 Jun 2008	39°06′N	75°53′W	A1(2), A8(3), A14(1)	6	N
MD (C)	Three Bridges Branch	Queen Anne's	12 Jun 2008	39°03′N	76°02′W	A1	7	N
MD (C)	Nassawango Creek	Worcester	9 Jul 2007	38°14′N	75°28′W	A1	4	N
Europe								
GB	Clifton Pond, Attenborough	Nottinghamshire	Apr 2006	52°54′N	1°14′W	A1	3	
NL	Nederhardinxveld	Zuid-Holland	13 Jan 2010	51°50′N	4°52′E	A1	7	
BE	Zonhoven	Flemisch Region	Jan 2008	50°59′N	5°22′E	A1	5	
FR	Auxances, Migné-Auxances	Poitou-Charentes	13 Jul 2006	46°37′N	0°18′E	A1	2	
FR	Vouneuil sous Biard	Poitou-Charentes	13 Jul 2006	46°34′N	0°16′E	A1	1	
FR	Jazeneuil	Poitou-Charentes	13 Jul 2006	46°27′N	0°04′E	A1	3	
FR	Lac d'Ilay	Franche-Comté	7 Aug 2007	46°37′N	5°54′E	A1	3	
DE	Rhine, Breisach	Baden-Württemberg	10 Aug 2005	48°01′N	7°34′E	A1	3	
DE	Naab, Pielenhofen	Bayern	29 Jun 2004	49°04′N	11°57′E	A1	2	
IT	Ticino, Pavia	Lombardia	Mar 2008	45°10′N	9°09′E	A1	1	
IT	Cherio, Borgo di Terzo	Lombardia	Mar 2008	45°43′N	9°53′E	A1	1	
CZ	Záluží u Litvínova	Ústí nad Labem Region	Jun 2007	50°33′N	13°36′E	A1	4	
CZ	Cítov, Mělník	Central Bohemia	12 Oct 2005	50°21′N	14°26′E	A1	3	
CZ	Lhota	Central Bohemia	Jul 2005	50°15′N	14°40′E	A1	4	
CZ	Smečno	Central Bohemia	9 Apr 2006	50°12′N	14°02′E	A1	2	
CZ	Hracholusky, Čerňovice	Plzeň Region	25 Jun 2006	49°48′N	13°06′E	A1	2	
CZ	Starý Klíčov, Domažlice	South Bohemia	23 Oct 2005	49°24′N	12° 58′E	A1	4	
CZ	Soběslav	South Bohemia	23 Aug 2007	49°15′N	14°43′E	A1	1	
CZ	Malše, České Budějovice	South Bohemia	12 Sep 2005	48°58′N	14°29′E	A1	7	
CZ	Prudník, Osoblaha	Silesia	27 Oct 2006	50°18′N	17°43′E	A1(8), A4(4)	12	
PL	Jegrznia River, Wojdy	Podlaskie	3 May 2008	53°43′N	22°42′E	A1	5	
PL	Lake Spore, Szczecinek*	Zachodnio-pomorskie	Sep 2000	53°47′N	16°42′E	A1	1	
PL	Vistula Lagoon, Elblag*	Warmińsko-Mazurskie	Sep 2001	54°16′N	19°20′E	A1	1	
HU	Ipoly, Letkés	Pest	Oct 2008	47°53′N	18°46′E	A1	3	
HU	Danube-Ipoly, Szob	Komárom-Esztergom	18 Oct 2008	47°49′N	18°50′E	A1	1	
HU	Danube, Göd	Pest	Oct 2006, 2008	47°41′N	19°07′E	A1	10	
HU	Bóni-fok, Bogyiszló	Tolna	14 Oct 2006	46°22′N	18°47′E	A1	2	

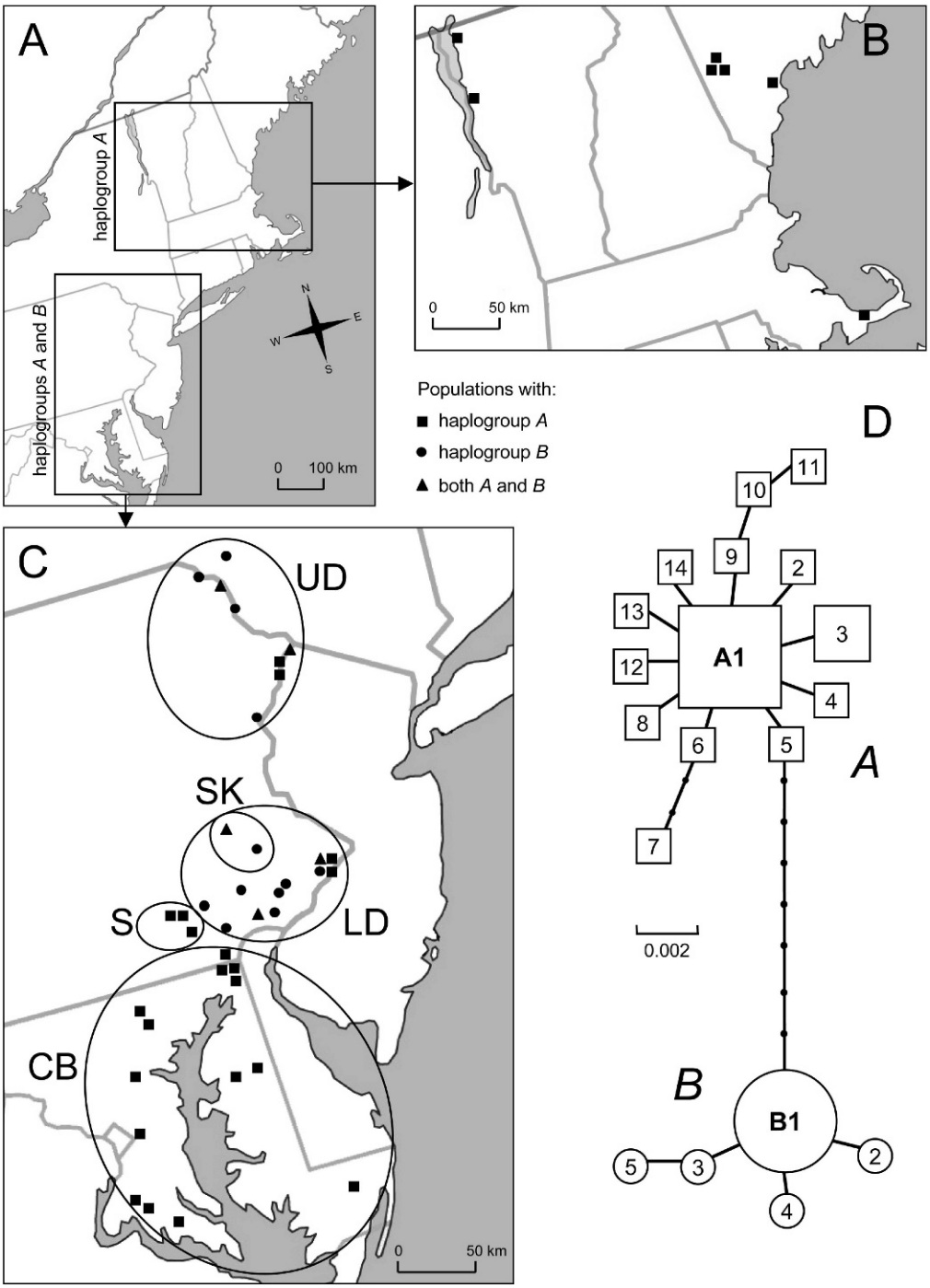

FIG. 2. Locations of studied *Orconectes limosus* populations in the USA (A); in Maine, Vermont and Massachusetts (B); and in Pennsylvania, Maryland, and New York (C). Rectangles in panel A indicate the presence of the 2 haplogroups. Populations of *O. limosus* from the main drainages in Pennsylvania and Maryland are delimitated by ovals in panel C: upper Delaware River Basin (UD), lower Delaware River Basin (LD), Schuylkill River Basin (SK), Susquehanna River Basin (S), and the Chesapeake Bay Basin (CB). A distant population from Nova Scotia, Canada (only haplogroup A) was not included on the map. D.—Network of cytochrome c oxidase subunit I (COI) haplotypes detected in *O. limosus* individuals from Europe and North America. The sizes of squares and circles in the network correspond to their frequency in populations (large symbols = dominant haplotypes A1 and B1, found in 44 and 16 populations, respectively; medium symbol = haplotype A3, found in 9 populations; small symbols = haplotypes found in 1 or 2 populations).

each primer, 0.625 U of Taq polymerase (Promega), and 0.5 μL of template (~100 ng DNA). After an initial denaturation step of 5 min at 95°C, we applied 35 cycles of 50 s at 95°C, 50 s at 55°C, and 50 s at 72°C with a final extension for 5 min at 72°C. Last, we used Exonuclease I (New England Biolabs, Ipswich, Massachusetts) and Shrimp Alkaline Phosphatase (Fermentas, Burlington, Ontario) to purify PCR products, with incubation for 1 h at 37°C followed by 20 min at 80°C. We sequenced purified products on an ABI PRISM 3130 capillary sequencer using LCO primers and the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California). We truncated sequences to 639 bp, and submitted sequences representing each haplotype recorded in each studied population to GenBank (accession numbers JF911534–JF911620).

We analyzed results with the software package MEGA (version 4.0; Tamura et al. 2007). All sequences could be aligned unambiguously by eye. We constructed a haplotype network with the program TCS (version 1.21; Clement et al. 2000). We used the Kimura-2-parameter model to calculate average pairwise divergence between 2 haplotype clusters observed within the species.

Results

In total, 19 haplotypes were detected in populations of *O. limosus* from North America and Europe. Haplotypes were grouped in 2 well separated clusters (*A* and *B*; Fig. 2D). The genetic distance between central haplotypes of these clusters (A1 and B1) was 1.3%. Most of the haplotype variation (18 haplotypes) was concentrated in North American populations (Table 1); only 2 haplotypes were found in Europe. Most haplotypes were rare in North American populations and occurred in 1 or 2 populations. Only 3 haplotypes were more common (central haplotypes A1 and B1 in 44 and 16 populations, respectively, and A3 in 9 populations).

As expected, distributions of main haplotype groups differed within the native range (Fig. 2A–C). The number of haplotypes also differed among regions, but this difference could have been partly the result of differences in sampling effort. Twelve haplotypes (most with very restricted distributions) were recorded in southeastern Pennsylvania and northern Maryland; 7 of these occurred in the Lower Delaware region (southern Pennsylvania). Three haplotypes were found in the Upper Delaware region, and 4 were found further north in the species' present range (Table 1, Fig. 2C).

Haplotype A1, dominant in Europe, was present in Nova Scotia (3 of 9 individuals), Maine (16/16), Vermont (1/2), northern Pennsylvania (26/36), and Maryland (20/44). The 2nd most common haplotype, B1, was mainly found in southern Pennsylvania (50/70) but also occurred in northern Pennsylvania (9/36) and New York (single available sequence). The remaining haplotypes of the haplogroup B (B2–B5) were found mostly in southeastern Pennsylvania, but some also occurred in northeastern Pennsylvania (Table 1, Fig. 2C).

In contrast to populations in North America, European populations had almost uniform sequences at the analyzed COI fragment. The vast majority of European individuals (89 of 93 sequences, representing all sampled populations) carried an identical haplotype (A1 in Fig. 2D). In 4 of 12 individuals from a single locality (Prudník, Czech Republic), another haplotype (A4) was found, which differed by a single point mutation from the common haplotype, A1. This rare haplotype was not observed in North American or other European localities.

Discussion

Colonization of Europe

Comparison of mtDNA diversity in invading European populations of *O. limosus* with mtDNA diversity in the species' native range confirms a substantial bottleneck in this species during its introduction to Europe. Most of the European populations analyzed shared a single haplotype, a pattern that is consistent with the scenario of colonization from a single source by a limited number of founders and is in agreement with historical accounts (McDonald 1893). The number of colonists allowed preservation of some diversity of nuclear markers (as observed by allozyme and microsatellite data; Filipová et al. 2009, Hulák et al. 2010), but was not sufficient to import substantial diversity at the mtDNA level. Mitochondrial markers are more susceptible than nuclear markers to the effects of genetic drift. The effective population size for mitochondrial loci is 4× smaller than for nuclear loci because of differences in ploidy and inheritance (Birky et al. 1989). Furthermore, the source population itself probably had limited mtDNA diversity.

The alternative scenario of multiple *O. limosus* introductions to Europe would explain the success of the species in colonizing various habitats and European regions with differing climates. However, this scenario was not supported by the data. Multiple introductions of *O. limosus* from an area with the same dominant haplotype cannot be completely excluded, but the concordance of low mtDNA diversity with the historical records of the species' introduction makes this scenario less likely. Unfortunately, our data did not allow us to pinpoint the most likely source region for the introduction. The haplotype A1, dominant in Europe, also was

dominant in many parts of the North American range of *O. limosus*, whereas the rare haplotype A4 was not found among North American samples. However, our data do suggest that the source region was not southeastern Pennsylvania (including the Lower Delaware) or northern Maryland.

Invasive populations of *P. clarkii*, another crayfish species introduced from North America to Europe, also had less COI variation than populations from its native range (Barbaresi et al. 2007). However, COI variation in European populations of *P. clarkii* was relatively high (6 haplotypes in 53 individuals from 10 populations), a result that corresponds to a scenario of multiple secondary introductions in Europe of individuals from different sources (Barbaresi et al. 2007). Substantial variation in COI haplotypes in European populations of *P. leniusculus* (Filipová et al. 2011, and unpublished data) probably also reflects multiple introductions during colonization. In contrast, mtDNA diversity of *O. limosus* in Europe suggests that <100 individuals from 1 source probably are the ancestors of all European *O. limosus* populations and that the success of its invasion was not limited by an introduction bottleneck.

Genetic diversity in North America

A much larger number of haplotypes was found in North America than in Europe. These haplotypes formed 2 genetically distinct haplogroups (Fig. 2D). Haplogroup A was widely distributed, whereas the center of diversity of haplogroup B was in southeastern Pennsylvania. Only the common haplotype B1 and a rare haplotype B4 were found away from that region (Table 1, Fig. 2C). At present, the 2 haplogroups of *O. limosus* are not geographically separated.

Two star-shaped clusters in the haplotype network suggest recent rapid expansions and differentiation from central haplotypes. The divergence is comparable to that observed among some closely related lineages of the *Orconectes virilis* (Hagen, 1870) species complex (Mathews et al. 2008, Filipová et al. 2010) and within the European white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) (Trontelj et al. 2005). Both of these taxa are thought to have diversified during the Pleistocene (Trontelj et al. 2005, Mathews et al. 2008).

Lower genetic variation in the northern part of the present North American range of *O. limosus* than in the southern part might be the result of introduction bottlenecks and founder effects caused by recent expansions. Originally, *O. limosus* is thought to have inhabited the Piedmont and Coastal Plain Physiographic Provinces from Virginia northward to New Jersey (Ortmann 1906, Bouchard et al. 2007), but considerable uncertainty exists regarding the native range of the species (Ortmann 1906, Crocker 1979, Bouchard et al. 2007). Ortmann (1906) observed that the range of *O. limosus* did not seem to reach far north in Pennsylvania at the beginning of the 20th century and speculated, as did Bouchard et al. (2007), that the species may have spread north and west of its range in southeastern Pennsylvania through artificial canals or with the assistance of humans. Bouchard et al. (2007) also hypothesized that *O. limosus* could have reached the Upper Delaware River system from the north via the Delaware and Hudson Canal in southeastern New York and northeastern Pennsylvania, a scenario that may explain the apparent absence of the species in the central part of the river (Ortmann 1906, Bouchard et al. 2007).

Further north (New England and Nova Scotia), *O. limosus* is thought to have been introduced to some areas. Maine and Nova Scotia are considered to have been colonized recently (McAlpine et al. 1991, Souty-Grosset et al. 2006, Lambert et al. 2007). Most individuals from the single population sampled in Nova Scotia shared haplotype A3 with individuals found much further south in several brooks in northern Maryland, in the lower Delaware River, and in some smaller watercourses in Pennsylvania (Table 1). The conspicuous sharing of the dominant haplotype between invaded areas in Europe and North America suggests that the individuals that originally invaded those areas may have come from the same general source area.

The construction of artificial canals connecting watersheds and human introductions could explain the recent spread of the species from its original range and some of the patterns of haplotype distribution (e.g., lower genetic diversity to the north). However, these patterns also could reflect more ancient processes, in particular, recolonization after the last ice age. Ortmann (1906) and Rhoades (1962) suggested that *O. limosus* could have persisted during the Wisconsin glaciation in a refuge in the lower Delaware River and Chesapeake Bay. The adjacent southeastern part of the Piedmont plateau in Pennsylvania and Maryland also is thought to be the last remnant of its former range in the Piedmont (Bouchard et al. 2007). The higher number of haplotypes detected in that area may support this view because regions in which a species has persisted for a long time are more likely to host high genetic diversity than are recently colonized regions.

However, gaps in the distributions of several aquatic animal and plant species, including *O. limosus* in the central part of the Delaware River (Bouchard

et al. 2007) and the white river crayfish *Procambarus acutus* (Girard, 1852) and other taxa in parts of New England (Smith 1982), also might be explained by the existence of a 2nd glacial refuge on the formerly exposed continental shelf, such as the one off southeastern New England proposed by Coddington and Field (1978). If *O. limosus* in such a refuge had reduced haplotype diversity relative to those in the southern refuge, colonization of northern regions from that source could have led to the patterns of genetic diversity observed today.

Further genetic analyses and more information about past human activities are needed to reconstruct the recent colonization history of *O. limosus* in its North American range. Analyses of nuclear markers (e.g., microsatellite markers; Hulák et al. 2010) would be desirable. However, samples consisting of substantially more individuals per population than was possible in our study would be needed. More detailed data will be important for the possible reassessment of the conservation status of *O. limosus* in the USA. The species has been categorized as 'currently stable' by Taylor et al. (2007), but many populations in its original range (e.g., in the southeastern part of the Piedmont Province) are threatened by aggressively spreading invasive crayfish species, such as *Orconectes rusticus* (Girard, 1852) and *O. virilis* (Bouchard et al. 2007, Kilian et al. 2010, Lieb et al. 2011a, b). These threatened populations seem to contain unique haplotypes that are not found elsewhere. For example, we found unique haplotypes of *O. limosus* in close proximity to dense populations of exotic crayfishes in the Susquehanna River drainage. *Orconectes limosus* appears already to have gone extinct in West Virginia, and invasive crayfish have probably contributed to its extirpation (Loughman et al. 2009, Swecker et al. 2010). The clear geographical pattern of *O. limosus* mtDNA diversity indicates that irreversible losses of genetic variation may occur even if the species itself is not endangered in other parts of its present range.

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CHAPTER II

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Allozyme variation in Czech populations of the invasive spiny-cheek crayfish *Orconectes limosus* (Cambaridae)

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ABSTRACT

Key-words: Orconectes limosus, *allozymes, genetic variation, Czech Republic*

The North American spiny-cheek crayfish, *Orconectes limosus*, was most probably introduced into Europe only once, in 1890. The size of the founding population was just 90 individuals. Low genetic variability resulting from a bottleneck effect during introduction might therefore be supposed in European spiny-cheek crayfish populations. On the other hand, the fast spread of *O. limosus* in Europe and colonisation of various habitats suggest that this species does not suffer from inbreeding depression due to an introduction bottleneck. We analysed 14 *O. limosus* populations from the Czech Republic using allozyme electrophoresis to evaluate the level of intra- and among-population genetic variation. Out of eight well-scoring allozyme loci chosen for detailed analysis, six were variable in studied populations, suggesting that sufficient variability was maintained during the introduction. Genetic differentiation of Czech populations of the spiny-cheek crayfish was relatively low and did not show any clear geographic pattern, probably due to long-range translocations by humans.

RÉSUMÉ

Variation des allozymes dans les populations de l'écrevisse américaine, *Orconectes limosus* (Cambaridae), en République tchèque

Mots-clés : Orconectes limosus, *allozymes, variation génétique, République tchèque*

L'écrevisse américaine, *Orconectes limosus*, a été très probablement introduite en Europe en une seule fois, en 1890. La taille de la population initiale était seulement de 90 individus. Suite à un goulet d'étranglement (bottleneck effect) pendant l'introduction, on pourrait s'attendre à une variabilité génétique réduite. Cependant, la dispersion rapide d'*O. limosus* en Europe et la colonisation d'habitats très variés indiquent que cette espèce ne souffre pas d'inbreeding causé par le goulet d'étranglement. Par électrophorèse des allozymes, nous avons analysé 14 populations d'*O. limosus* en République tchèque pour évaluer le taux de variation génétique intra- et inter-populationnel. Des huit loci choisis pour cette étude, six étaient variables dans les populations étudiées, suggérant qu'une variabilité suffisante a été maintenue lors de la première introduction. La différenciation génétique des populations tchèques de l'écrevisse américaine était assez basse et sans aucun pattern géographique, conséquence probable des translocations à grande distance effectuées par l'homme.

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INTRODUCTION

With the increasing number of species introduced into new territories, the need arises to understand the process of colonisation and factors influencing the distribution potential of those species. Apart from other factors (*e.g.*, adaptations for dispersal and the competitive ability of the species, the character of invaded habitats and communities, the presence or absence of predators and pathogens), the success of invasive taxa may depend on the genetic variability of introduced populations (Sakai *et al.*, 2001). High genetic variability is supposed to be advantageous in invading new areas, because in sexual species it allows more rapid evolution and adaptation to changing environmental conditions (Lambrinos, 2001; Sakai *et al.*, 2001). An example of a highly genetically diverse successful invader is the North American crayfish, *Procambarus clarkii*, in European waters (Barbaresi *et al.*, 2007). However, even organisms with very low genetic variability in their invasive populations can be excellent colonisers. In extreme cases, a single clone of a species may become a widespread invader, as is the case for the tropical alga *Caulerpa taxifolia*, invading a very large area of the North-western Mediterranean Sea (Jousson *et al.*, 1998), or the obligately parthenogenetic clone of the water flea *Daphnia* (interspecific hybrid of two North American lineages of the *D. pulicaria* complex) spreading in Africa (Mergeay *et al.*, 2006). In these cases, low genetic diversity does not obstruct the invader's spread and competition with genetically diverse indigenous species.

As mentioned above, North American crayfish are prominent among successful invasive animal groups in European continental waters. Several species have been introduced into the continent since the end of the 19th century, in attempts to replace lost native crayfish populations decimated by crayfish plague. Three of these invasive crayfishes – the signal crayfish (*Pacifastacus leniusculus*), the red swamp crayfish (*Procambarus clarkii*), and the spiny-cheek crayfish (*Orconectes limosus*) – are extremely widespread in Europe (Souty-Grosset *et al.*, 2006). They inhabit various types of habitats and successfully compete with native crayfishes; moreover, they serve as a vector of the oomycete *Aphanomyces astaci*, a pathogen of the crayfish plague (Vey *et al.*, 1983; Alderman *et al.*, 1990; Diéguez-Uribeondo and Söderhäll, 1993; Kozubíková *et al.*, 2009). Contact of infected American crayfish with native species often results in mass mortalities of the latter (Holdich, 1999; Söderhäll and Cerenius, 1999).

In our study, we focused on the genetic variation of the spiny-cheek crayfish, *Orconectes limosus* (Rafinesque, 1817). It was first introduced into Europe in 1890, when 90 individuals were released into a pond in Pomerania (currently western Poland) (McDonald, 1893; Kossakowski, 1966); this event has most probably been the only successful introduction of this species into Europe (Kossakowski, 1966). *O. limosus* has spread from the point of its original introduction to at least 20 European countries (listed in Holdich *et al.*, 2009), both naturally and through human-mediated translocations. Among those who spread non-native crayfish to both standing and running waters in Central Europe are especially anglers, owners of water bodies, and recreational scuba divers, usually being unaware of the negative impact of such activities on local ecosystems (Petrusek *et al.*, 2006).

Orconectes limosus is the most widespread non-indigenous crayfish in the Czech Republic. Its presence in the country was first confirmed in 1988 close to the border with neighbouring Germany (Hajer, 1989). However, the species was probably already present in the country in the 1960s; it most likely invaded the territory by migrating up the river Elbe from Germany (Kozák *et al.*, 2004; Petrusek *et al.*, 2006). Since the late 1980s, these crayfish have quickly spread over a large area of the Czech Republic, especially in the western part (Petrusek *et al.*, 2006). *O. limosus* can currently be found mostly in large watercourses, lower reaches of their tributaries, and in isolated standing waters such as flooded quarries, sandpits or ponds. The detailed distribution of the spiny-cheek crayfish in the Czech Republic has been reviewed by Petrusek *et al.* (2006) and Filipová *et al.* (2006). However, new scattered localities with the presence of this species are still being discovered. For example, *O. limosus* was found in 2006 in the south-west of the country in a large reservoir in the Bohemian Forest mountains (Beran and Petrusek, 2006), as well as in the north-east (Silesia), in the Prudník brook close to the border with Poland (Ďuriš and Horká, 2007; Kozubíková *et al.*, 2008).

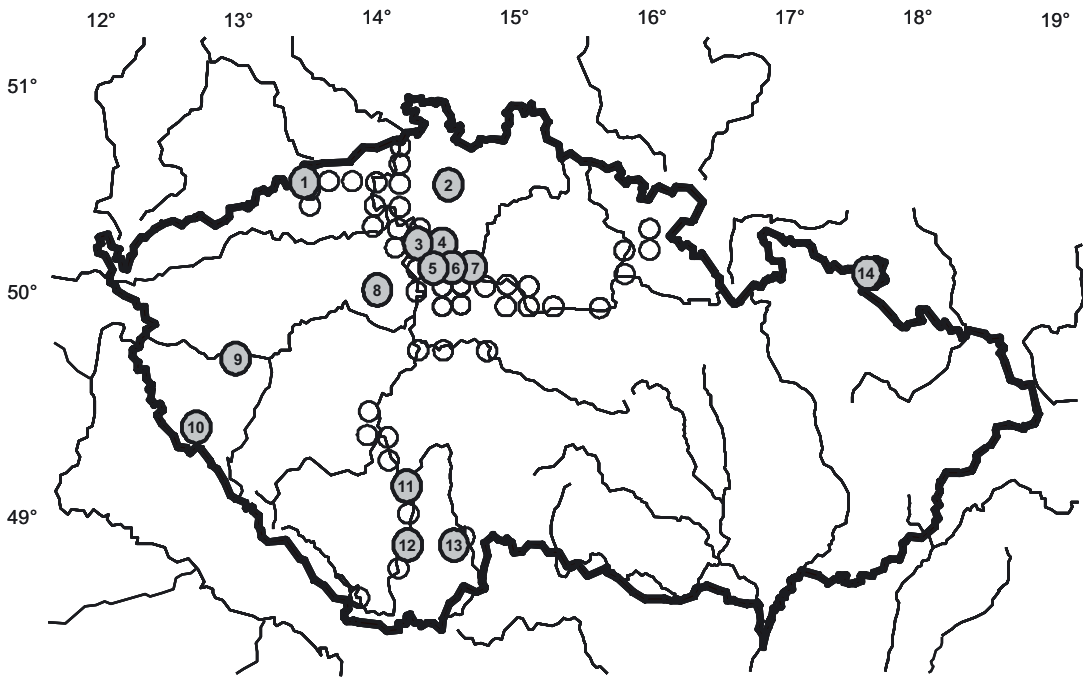

Figure 1
Distribution of O. limosus *in the Czech Republic (empty circles) and localities where individuals were sampled for the present study (grey circles with numbers, corresponding to the codes of localities in Table I).*

Figure 1
Répartition d'*O. limosus* en République tchèque (cercles vides) et sites de prélèvement des individus inclus dans cette étude (cercles gris avec les chiffres, ces chiffres correspondant aux codes des localités dans le Tableau I).

The aim of this study was to assess the level of genetic variability of selected populations of this species in the Czech Republic, using allozyme electrophoresis. We tested the hypothesis that sufficient genetic variation was maintained during the introduction of this species into Europe, so that allozyme markers can be used to analyse the genetic structure of these populations. Although the distribution of the spiny-cheek crayfish clearly suggests a large influence of long-range translocations within the Czech Republic, we wanted to test this by comparing the genetic and geographic distances of the studied populations.

MATERIALS AND METHODS

> *SAMPLING*

Crayfish were sampled between 2004 and 2007 from various types of localities – brooks, sandpits, lakes, reservoirs and flooded quarries (Figure 1, Table I), in parallel with a study focusing on the distribution of the crayfish plague pathogen in populations of American crayfish in the Czech Republic (Kozubíková *et al.*, 2009). Nearly all samples came from the western part of the country where this crayfish is most abundant; all these populations are assumed to have originated from the initial invasion through the Elbe (Petrusek *et al.*, 2006). An exception in our dataset was the population from the Prudník brook in Silesia, which had been colonised by individuals by upstream or downstream migration from Poland (Ďuriš and Horká, 2007).
Crayfish were mostly captured by hand or while scuba diving. After transporting in cooling boxes, individuals were stored in a deep freezer (at –80 °C). The tissue for analyses was then

Table I

List of sampled localities (codes correspond to numbers on the map in Figure 1), date of sampling and summary of population characteristics: numbers of examined individuals (n), heterozygosity expected non-biased (Hexp. n.b.) [%] and heterozygosity observed (Hobs.) [%], mean number of alleles per locus and Hardy-Weinberg exact probability.

Tableau I

Liste des localités échantillonnées (les codes correspondent aux chiffres notés dans la carte de la Figure 1), leur caractère, date de prélèvement et caractéristiques des populations : nombre d'individus examinés (*n*), hétérozygotie attendue corrigée pour le biais d'échantillonnage (Hexp. n.b.) [%] et hétérozygotie observée (Hobs.) [%], nombre moyen d'allèles par locus et probabilité de conformité à l'équilibre de Hardy-Weinberg.

Code	Name of locality	Locality character	Latitude (N)	Longitude (E)	Date of sampling	*n*	Hexp. n.b.	Hobs.	Alleles/locus	H-W exact probability
1	Záluží	retention pond	50° 33′	13° 36′	Jun. 2007	22	2.50	2.27	1.875	0.007
2	Stará pískovna (Provodín)	sandpit	50° 37′	14° 36′	13 Sep. 2004	10	2.10	1.38	1.75	0.257
3	Cítov (Horní Počaply)	sandpit	50° 21′	14° 26′	12 Oct. 2005	8	2.99	2.59	2	0.216
4	Pšovka (Lhotka)	brook	50° 23′	14° 33′	4 Jun. 2005	10	2.30	1.50	1.75	0.437
5	Kojetice	quarry	50° 14′	14° 31′	20 Jul. 2005	20	2.74	2.38	2	0.172
6	Proboštská jezera (St. Boleslav)	sandpit	50° 12′	14° 39′	3 Sep. 2005	17	2.00	2.28	1.75	0.420
7	Lhota	sandpit	50° 14′	14° 40′	Jul. 2005	35	1.90	1.41	2.25	0.162
8	Smečno	pond	50° 11′	14° 2′	9 Apr. 2006	21	2.55	2.62	1.75	0.128
9	Hracholusky (Přovany)	reservoir	49° 47′	13° 6′	25 Jun. 2006	11	2.17	1.71	1.875	0.293
10	Starý Klíčov (Mrákov)	quarry	49° 23′	12° 57′	23 Oct. 2005	20	3.19	2.88	2	0.131
11	Kořensko (Neznašov)	reservoir	49° 14′	14° 22′	26 Apr. 2004	11	2.36	2.50	1.75	0.249
12	Malše (České Budějovice)	river	48° 58′	14° 29′	12 Sep. 2005	12	1.30	1.04	1.375	0.133
13	Zlatá Stoka (Třeboň)	channel	49° 0′	14° 46′	19 Jul. 2006	14	1.33	1.25	1.625	0.007
14	Prudník (Slezské Pavlovice)	brook	50° 17′	17° 43′	27 Oct. 2006	11	2.79	1.82	1.875	0.181

dissected from legs or claws of the captured crayfish. Altogether, 222 individuals of *O. limosus* from 14 populations were analysed.

> ALLOZYME ELECTROPHORESIS

Horizontal cellulose acetate electrophoresis was used for the genetic analyses as described in Hebert and Beaton (1993). This was carried out in a Tris-Glycine buffer system (pH = 8.5) on 76 × 76 mm cellulose acetate plates (Titan III, Helena Laboratories). A small amount of tissue was dissected from crayfish legs or claws and homogenised with a plastic rod in about 10 μL of distilled water. The tissue of one crayfish individual was used as a standard in all analyses to assure comparable scoring. In each run, eleven animals and one standard, loaded in one row, were analysed.

Overall, seventeen enzymes were tested. Some of them did not show sufficient activity for routine screening and were therefore excluded from the analyses: α-amylase (AMY, EC 3.2.1.1), fumarate hydratase (FUM, EC 4.2.1.2), hexokinase (HEX, EC 2.7.1.1), xanthine dehydrogenase (XDH, EC 1.1.1.204), alcohol dehydrogenase (ADH, EC 1.1.1.1), α,α-trehalase (TRE, EC 3.2.1.28), isocitrate dehydrogenase (IDH, EC 1.1.1.42), malate dehydrogenase $NADP^+$ (ME, EC 1.1.1.40) and adenylate kinase (AK, EC 2.7.4.3). Furthermore, we did not include aspartate amino transferase (AAT, EC 2.6.1.1), although it scored well, as it showed very low migration speed under the conditions used for other enzymes.

Eight enzyme loci were finally selected for further analyses: glucose-6-phosphate isomerase (GPI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 5.4.2.2), mannose-6-phosphate isomerase (MPI, EC 5.3.1.8), malate dehydrogenase (two loci, MDH 1 and MDH 2, EC 1.1.1.40), arginine kinase (ARK, EC 2.7.3.3), lactate dehydrogenase (LDH, EC 1.1.1.27) and aldehyde oxidase (AO, EC 1.2.3.1). The most common allele for each locus was designated M (medium). Other alleles were labelled corresponding to their relative mobility to the M-allele: F (fast), S (slow), or S^- (very slow).

Allelic frequencies, observed and expected heterozygosities, F statistics (Weir and Cockerham, 1984), and genetic distances were calculated in Genetix 4.03 (Belkhir *et al.*, 1996). GenAlEx 6.1 (Peakall and Smouse, 2006) was used to test whether genotypic frequencies at the studied loci are consistent with Hardy-Weinberg expectations. Nei's genetic distance (Nei, 1978) was calculated to estimate levels of genetic distance between tested populations. Based on these results, a UPGMA dendrogram was created in Statistica 6.1 (StatSoft, Tulsa, USA) to depict similarity among studied populations. The relationship between genetic (Nei's) and geographic (log-transformed) distances between populations was tested by the Mantel test, comparing the respective pairwise distance matrices in the software zt (Bonnet and Van de Peer, 2002).

RESULTS

Out of eight loci used in our analyses, two enzymes (LDH and AO) showed no variability; the remaining six (GPI, PGM, MPI, MDH 1, MDH 2 and ARK) were polymorphic, *i.e.*, with more than one detected allele. However, in MDH 1 one of the two detected alleles was very rare (less than 1%). The most variable locus was PGM, with four different alleles detected; the slowest of them, S^-, was relatively rare (5.5%). In four enzymes (GPI, MDH 2, MPI and ARK), three different alleles could be distinguished (Table II).

A summary of population characteristics is shown in Table I. All 14 populations analysed in our study were polymorphic at two or more loci. The highest average number of alleles per locus was in the population from Lhota (2.25 alleles/locus), followed by populations from Cítov, Kojetice and Starý Klíčov (2 alleles/locus). The lowest average number of alleles per locus (1.38) was in the population from the Malše River.

Table II

Allele frequencies [%], number of detected alleles and mean number of alleles per population observed in eight studied loci in 222 individuals of the spiny-cheek crayfish from the Czech Republic.

Tableau II

Fréquences alléliques [%], nombre d'allèles détectés et nombre moyen d'allèles par population, observés dans huit loci chez 222 individus de l'écrevisse américaine en République tchèque.

Locus	Allele frequency [%]				Number of detected alleles	Mean number of alleles/population
	F	M	S	S^-		
GPI	38.9	19.7	41.4		3	2.71
PGM	18.8	11.4	64.4	5.5	4	2.92
MPI	0.1	83.6	16.3		3	1.64
MDH 1		99.4	0.6		2	1.07
MDH 2	48.0	18.1	33.8		3	2.92
ARK	1.4	95.7	2.9		3	1.35
LDH		100			1	1
AO		100			1	1

The observed heterozygosity of populations was consistent with expected values in most cases (Table I), and the loci studied were in good agreement with Hardy-Weinberg expectations in most populations. However, two of the studied populations, Zlatá stoka and Záluží, exhibited significant deviations from the Hardy-Weinberg equilibrium, both showing heterozygote deficiencies.

These studied Czech spiny-cheek crayfish populations were significantly genetically structured, with a mean F_{ST} value for all loci of 0.160. Nei's genetic distance between populations varied from 0.003 (between populations from Lhota and Malše) to 0.20 (between populations from Zlatá Stoka and Pšovka). The geographically distant population from Prudník (Silesia) did not markedly differ from other populations; it was genetically closest to the population from Záluží, located more than 290 km away.

The UPGMA dendrogram (Figure 2) did not show any apparent clustering. In some cases, even very distant localities were similar, such as the pairs Kořensko and Záluží (160 km) or Lhota and Malše (147 km). The relationship between geographic and genetic distances among populations was not significant (Mantel test, $p = 0.126$); this pattern did not change if the Prudník population (Silesian region), with different colonisation history, was excluded.

Some population pairs could have been expected to be more similar to each other than the rest of the studied Czech *O. limosus* populations, as one was the source of crayfish for the other: the population in Klíčov was founded by individuals from the Hracholusky Reservoir, and the sandpit Lhota was supplied with crayfish from the sandpit Proboštská jezera. However, we did not observe any substantially higher similarity between these populations in comparison with the others.

DISCUSSION

As Dlugosch and Parker (2008) showed, for invasions where a single introduction occurred, allelic richness is generally lower in introduced populations than in native ones. Moreover, reductions in genetic diversity tend to be inversely correlated with the size of the founder population (Merilä *et al.*, 1996). Although the European populations of *O. limosus* were apparently founded just once and by a relatively small number of individuals (at least in comparison with other widespread invasive crayfish in Europe, *P. leniusculus* and *P. clarkii*), the presence of several variable enzyme loci in the studied *O. limosus* populations suggests that the bottleneck effect was not very dramatic, and sufficient variation was retained during the introduction of this species into Europe. This is also supported by its rapid spread and presence in various habitats.

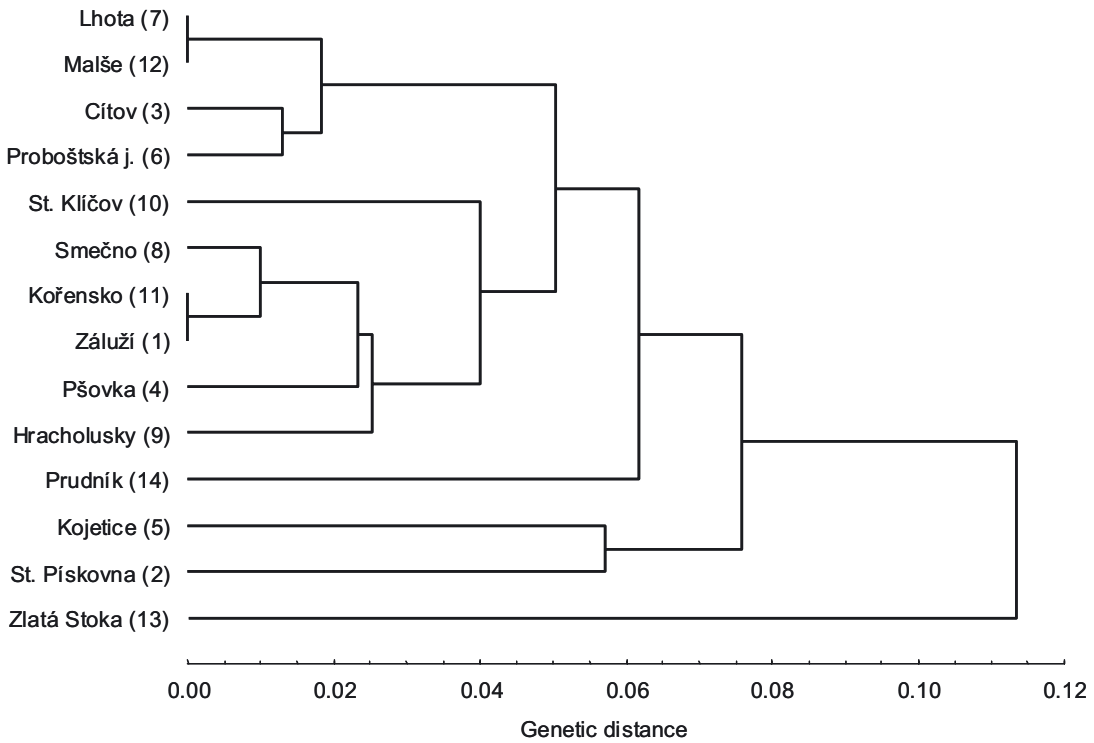

Figure 2
UPGMA dendrogram, using Nei's genetic distance (Nei, 1978), showing genetic similarity of O. limosus *populations included in our study (numbers correspond to the codes of localities in Table I).*

Figure 2
Dendrogramme UPGMA basé sur la distance génétique de Nei (Nei, 1978), montrant la similarité génétique des populations d'*O. limosus* impliquées dans notre étude (les chiffres correspondent aux codes des localités dans le Tableau I).

Nevertheless, the introduction may have resulted in some reduction in genetic variation, as the Czech spiny-cheek crayfish populations showed relatively low allozyme variation in comparison with other previously studied crayfishes such as *Astacus astacus* (Fevolden and Hessen, 1989), *Parastacoides tasmanicus* (Hansen *et al.*, 2001) and *Austropotamobius pallipes* (Lörtscher *et al.*, 1998; Largiadèr *et al.*, 2000). However, the variation we observed was still higher than in several other crayfishes: *A. leptodactylus* (Agerberg, 1990), and ten species of *Procambarus* and two species of *Cambarus* (Brown, 1981; Busack, 1989), in which an almost complete absence of variation was recorded, supposedly due to introduction bottlenecks.

Despite repeated bottlenecks during the colonisation of Czech waters by *O. limosus*, genetic variability has also been maintained in populations at the edge of the species' distribution in the country. Interestingly, the observed allozyme variation was usually higher in isolated populations (which must have been founded by men) than in those from rivers or brooks where crayfish may have dispersed naturally (Table I).

Our analyses did not show any obvious correlation between geographic distance and genetic similarity of the studied populations. This can be explained by human-mediated translocations of the spiny-cheek crayfish into the Czech Republic that influenced the genetic structure of the species. We originally supposed that the geographically distant population in the Prudník brook in Silesia might differ from the remaining Czech populations, as the individuals originated from a different region and stochastic events could have led to substantially different allele frequencies or private alleles. However, the allozyme variation in this population was similar to the others, suggesting that most alleles brought to Europe were transferred into

newly established populations of the species. Nevertheless, an analysis of the mitochondrial gene for cytochrome *c* oxidase subunit I (COI) of European *O. limosus* (Filipová, 2008) showed that the Prudník population differed from the rest of the analysed Czech populations in the presence of a rare haplotype found uniquely in several individuals from this population. Our results did not prove that pairs of populations in which one was founded by individuals from the other were genetically closer to each other. This might be due to either insufficient sampling or processes that influenced allele richness and frequency at the newly colonised localities, such as founder effects during introduction or subsequent genetic drift.

Our results on allozyme variation were useful in the selection of individuals and populations for analyses of other genetic markers. While sequencing of the mitochondrial COI gene, even from crayfish carrying unusual alleles, did not reveal any variation in Czech populations (with the exception of Prudník mentioned above), selection of individuals differing at allozyme loci simplified the screening for variable microsatellite markers in this species by cross-species amplification (Hulák *et al.*, 2010). Initial testing of ten microsatellite loci in one Hungarian and two Czech populations (including the one from Starý Klíčov analysed in this study) has subsequently confirmed that populations of the spiny-cheek crayfish are indeed genetically diverse (Hulák *et al.*, 2010). In the future, these variable nuclear markers may allow more detailed population-level studies of this invasive crayfish.

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CHAPTER III

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Genetic variation of invasive European populations of signal crayfish, *Pacifastacus leniusculus*

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Abstract

The signal crayfish *Pacifastacus leniusculus* is native in North America, where three subspecies are recognised. Since 1959, the species has been introduced to Europe several times; altogether more than 60 000 individuals have been imported. Secondary introductions across the continent followed, resulting in its current presence in at least 25 European countries. Previous studies suggested that more subspecies are found in Europe; this hypothesis seems supported by the presence of at least two subspecies in the source area of most individuals imported to Europe. Our aim was to investigate genetic variation in invasive European populations of this species, and their taxonomic status. We obtained partial sequences of the mitochondrial gene for cytochrome *c* oxidase I (COI) of 348 signal crayfish from 70 European populations, and also of 32 individuals from six North American populations. These data were compared to sequences representing the three subspecies known from America (from individuals sampled in the Klamath River Basin). Our results suggest that despite substantial variation at the analysed gene, all studied European individuals fall within *P. leniusculus leniusculus*. In total, 27 haplotypes were observed in Europe, with relatively high average pairwise divergences among four main clades, reaching up to 3%. The distribution of COI haplotypes in Europe, four of which have been very common and widespread, did not show any clear geographic pattern; this corresponds to numerous secondary introductions of signal crayfish around the continent. Most individuals additionally analysed from American populations were assigned to the *P. l. leniusculus*. However, six samples from the Chehalis River (Washington) represent a distinct lineage, with 6.2% to 7.0% divergence to the three already recognised ones. This shows that genetic variation within signal crayfish is higher than previously detected and more studies are needed to investigate relationships of cryptic groups within populations attributed to *P. leniusculus*.

Key-words: *Pacifastacus leniusculus*, invasive crayfish, genetic variation, subspecies, Europe, North America

Introduction

Since 1890, several non-indigenous crayfish species have been introduced to Europe to substitute for native crayfish populations decimated by the pathogen of the crayfish plague (Holdich et al. 2009, Souty-Grosset et al. 2006). Nowadays, the most widespread non-indigenous crayfish species in Europe is the North American signal crayfish *Pacifastacus leniusculus* (Dana, 1852) (Holdich et al. 2009). This species, previously considered to be immune to crayfish plague, was introduced for aquaculture and stocking. However, signal crayfish represent a serious danger for native European crayfish species, both by the transmission of the crayfish plague pathogen *Aphanomyces astaci* (of which they are natural hosts), and by direct competition (Holdich 1999, Westman and Savolainen 2001, **chapter VI**). Its good competitive abilities have also been documented in North America where the species contributed to the extinction of its congener *Pacifastacus nigrescens* in the San Francisco Bay area and to the decline of *P. fortis* in northeastern California (Bouchard 1977, Light 1995, Riegel 1959).

The native range of *P. leniusculus* is in the northwestern part of the USA in Washington, Oregon, Montana, Idaho, Nevada, Utah, and also in British Columbia, Canada (Bondar et al. 2005, Hobbs 1989, Souty-Grosset et al. 2006). Three subspecies of *Pacifastacus leniusculus* are currently recognized: *P. l. leniusculus* (Dana, 1852), *P. l. klamathensis* (Stimpson, 1857) and *P. l. trowbridgii* (Stimpson, 1857). They were initially considered different species and their status has later been changed to subspecific level. However, this division has often been questioned (Agerberg and Jansson 1995, Larson and Olden 2011, Miller 1960, Riegel 1959). The three subspecies were characterized by differences in morphology (e.g., rostrum length, rostral and postorbital spines or chelipeds), but these morphological features, as well as subspecies distributional ranges, substantially overlap (Larson and Olden 2011, Miller 1960). Attempts to distinguish the signal crayfish subspecies by allozyme analyses were also ambiguous (Agerberg and Jansson 1995). Lately, Sonntag (2006) detected three distinct mitochondrial lineages of *P. leniusculus*, which correspond to the three known subspecies, in the Klamath River Basin (California and Oregon, USA). However, it should be noted that the area investigated by this author was much smaller than the whole range of *P. leniusculus* in North America.

Signal crayfish have been introduced to several localities in the western USA, and also to Japan and Europe (Henttonen and Huner 1999, Riegel 1959, Usio et al. 2007). The status of the species in California, an important source region for introductions to Europe, is not clear. While Riegel (1959) mentions that only *P. l. klamathensis* is native to the area, Hobbs (1989) lists all three subspecies as native. This confusion might be due to the fact that the species has been introduced to California multiple times from different sources.

Particularly complicated is the introduction history of signal crayfish to Lake Tahoe, which provided most signal crayfish imported to Europe (Abrahamsson 1973b, Goldman et al. 1975). This lake and its tributaries in California were probably first stocked in 1895 by 56 signal crayfish from the Klamath River (Abrahamsson and Goldman 1970). In 1909, crayfish probably originating in coastal streams of Oregon were introduced to the lake (Goldman 1973) and in 1916, individuals suggested to be *P. l. klamathensis* were brought from the Klamath River (Riegel 1959). In 1912, California Fish and Game Commission (Santa Cruz County, California) received signal crayfish from the Columbia River (at the border between Oregon and Washington), which were then distributed to other parts of the state (Riegel 1959). By the mid-20th century, northern California has been most probably inhabited by all three subspecies (Miller 1960), and Lake Tahoe harboured individuals of at least two but possibly all three subspecies. Riegel (1959) mentioned *P. l. klamathensis* and *P. l. leniusculus* to be found in the area but considered *P. l. trowbridgii* synonymous with *P. l. leniusculus* (and thus would not have reported it separately); Miller (1960) suggested that both *P. l. leniusculus* and *P. l. trowbridgii*, or their intergrades may have been introduced to the lake. All of the above-mentioned introductions to California may have contributed to subsequent stockings to European waters (Agerberg 1993).

Signal crayfish were first brought to Europe in 1959 when 60 individuals from Natoma River, American River and Sacramento River drainages (California) were introduced to Sweden (Lake Träsksjön; Agerberg 1993). This trial stocking was successful, and large numbers of signal crayfish were subsequently imported from America and widely distributed around the continent (Henttonen and Huner 1999, Souty-Grosset et al. 2006). In 1969, a total of 58 000 specimens were brought from Lake Tahoe to Sweden (Brinck 1977, Fürst 1977, Goldman et al. 1975) and 6500 signal crayfish were introduced from Californian lakes Tahoe and Hennessey to Finland (Skurdal et al. 1999). California was also source of individuals for illegal releases of signal crayfish to Austria in 1969 (Henttonen and Huner 1999). In 1974, *Pacifastacus leniusculus* was brought from Lake Tahoe and Lake Donner to central part of France (Arrignon et al. 1999), the country also received individuals from Oregon (Souty-Grosset et al. 2006). Another locality, Pitt Lake in British Columbia (Canada), provided about 200 specimens which were introduced in 1975-1980 to Sweden (Agerberg and Jansson 1995).

Most crayfish used for subsequent introductions across Europe were raised in a hatchery Simontorp in Sweden (Henttonen and Huner 1999, Skurdal et al. 1999). Until 1976, about 800 000 signal crayfish from this hatchery were stocked into natural waters in Sweden (Fürst 1977). Until 1977, the country provided about 250 000 signal crayfish that were distributed to other European countries (Brinck 1977). For example, Poland received 30 000 individuals from Sweden (Kossakowski and Kossakowski 1978) and about 40 000 individuals

of the same origin were released in Luxembourg (Arrignon et al. 1999). Sweden provided crayfish also to Finland (Westman and Savolainen 2001), Spain (Gutiérrez-Yurrita et al. 1999), France (Arrignon et al. 1999), Austria (Abrahamsson 1973a) and the Czech Republic (Ircing and Prášil 1980). Germany received signal crayfish from Sweden but also from Austria (Dehus et al. 1999). Further secondary introductions within most of the above-mentioned countries, and further natural as well as human-mediated dispersal of signal crayfish followed, and the species is now present in at least 25 European countries (Souty-Grosset et al. 2006, Holdich et al. 2009).

The taxonomic status of signal crayfish imported to Europe from North America is unclear. Hobbs (1989) mentioned that only *P. l. leniusculus* had been introduced to Europe. However, based on analysis of allozyme markers in Swedish populations and three American populations representing the three known subspecies, Agerberg and Jansson (1995) suggested that both *P. l. leniusculus* and *P. l. trowbridgii* might be found in Sweden, while *P. l. klamathensis* did not seem to be present. Grandjean and Souty-Grosset (1997) also noted that high heterogeneity observed at mitochondrial markers in several French populations could be due to the presence of more subspecies. Moreover, as at least two subspecies are present in Lake Tahoe (Miller 1960, Riegel 1959) that provided most individuals introduced to Europe, thus, presence of more than one signal crayfish lineage at the continent seemed likely. The status of signal crayfish introduced to Japan is also unresolved. Between 1926 and 1930, signal crayfish were brought to Japan from the Columbia River basin, northwestern USA (Usio et al. 2007), and Hobbs (1989) noted that both *Pacifastacus l. leniusculus* and *P. l. trowbridgii* were introduced there. However, based on morphological examination, the subspecific status of signal crayfish in Japan could not be assessed (Kawai et al. 2003).

Although several studies dealt with some aspects of genetic variation in European signal crayfish (Agerberg 1990, Agerberg and Jansson 1995, Grandjean and Souty-Grosset 1997, Filipová et al. 2011a), these investigated only a few individuals or populations. Thus, the aim of the present study was to investigate genetic variation in European populations of signal crayfish over a large scale, and assess their lineage diversity. Given the discrepancy of previous genetic studies and the fact that more subspecies are found in the source areas of numerous signal crayfish introductions to Europe, we tested whether we detected lineages that can be linked to different subspecies of *P. leniusculus*. We also studied distributional patterns of common haplotypes, as these might reflect the colonization history of the species on the continent. This was already shown for other non-indigenous crayfish species in Europe, the spiny-cheek crayfish *Orconectes limosus* (Filipová et al. 2011b) and the red swamp crayfish *Procambarus clarkii* (Barbaresi et al. 2003, 2007).

Given the introduction history summarised above, various scenarios resulting in different patterns of genetic diversity in introduced European populations are possible. The mixed origin of crayfish in Lake Tahoe may have resulted in high variation in signal crayfish in the lake and subsequent high variation in individuals imported in large numbers to Europe. Alternatively, establishment of signal crayfish population in Lake Tahoe may have been associated with multiple introduction bottlenecks, and resulted in impoverished genetic variation not only in local signal crayfish but also in those bred in Sweden and distributed over most Europe.

We analysed cytochrome *c* oxidase subunit I (COI) gene fragment of signal crayfish from 17 European countries and we compared our results with data from the Klamath River basin in North America (Sonntag 2006) which included COI sequences of individuals representing three known subspecies of *P. leniusculus*. We have also analysed additional individuals from six North American populations to investigate variation in other parts of its American range.

Material and methods

In total, 348 individuals of *Pacifastacus leniusculus* from 70 populations were collected from representative localities in 17 European countries, pooled into eight main regions (Table 1, Fig. 1). Although not all countries with wide presence of signal crayfish were covered to a similar extent, we managed to obtain samples across most of the area colonised by this species in Europe except Greece. Moreover, 32 individuals from six populations were analysed from North America (Table 1).

One or two legs of captured crayfish were preserved in 96% ethanol. Muscle tissue of one leg segment was dissected from each crayfish. Chelex extraction protocol was used to isolate total genomic DNA (as described e.g. in Filipová et al. 2011), and the resulting supernatant was stored at -20°C. The amplification was carried out in 25 µl volumes, in a reaction mix containing a PCR Buffer with 1.5 mM $MgCl_2$ (Promega, USA), 400 µM dNTPs, 1 µM each primer, 0.025 U of the Taq polymerase (Promega, USA) and 0.5 µl of the template (about 100 ng DNA). Universal primers HCO 2198 and LCO 1490 (Folmer et al. 1994) were used to amplify the analysed COI fragment. The amplification program consisted of an initial denaturation step of 5 min at 95°C, 35 cycles of 50 s at 95°C, 50 s at 55°C and 50 s at 72°C, with a final extension for 5 min at 72°C. Exonuclease I (New England Biolabs, USA) and Shrimp Alcaline Phosphatase (Fermentas, Canada) was applied to purify the PCR products, with subsequent incubation for 1 hour at 37°C followed by 20 min at 80°C. Resulting products were then sequenced on a capillary sequencer (ABI PRISM 3130) using

LCO primers and the BigDye v. 3.1 Terminator kit. Obtained sequences were truncated to a length of 600 bp available for most samples.

New data were compared to reference COI sequences of the three known subspecies of signal crayfish from the Klamath River basin, North America (Sonntag 2006). Their codes which correspond to Sonntag (2006) represent *Pacifastacus leniusculus leniusculus* (F M225L22, H M220L22, N M185L18, O M183L18, P M179L18, U M141L13), *P. l. klamathensis* (J M208L21, W M101L09, Z M076L07) and *P. l. trowbridgii* (AC M019L02, AD M018L02, AE M017L02) (Fig. 2). Sequences of *Pacifastacus gambelii* (SN8EL29) and *P. fortis* (SH09LS1) taken from Sonntag (2006) were used as outgroups.

All sequences could be unambiguously aligned in the software MEGA version 5.05 (Tamura et al. 2011). The same software package using Kimura 2-parameter model was then used to construct the neighbour-joining tree and to calculate the average pairwise divergence between detected clades of *P. leniusculus*. Parsimony network of the haplotypes detected in Europe was constructed in TCS version 1.21 (Clement et al. 2000), with 93% connection limit.

Results

In 348 European individuals analysed in our study, 27 different COI haplotypes, one to five per population, were found (Table 1, Fig. 2); these are labelled E1-E5, EA6, E7-E24, EA25 and E26-E27. When compared to reference sequences from North America, all of these European haplotypes fell within a highly diverse lineage of *P. leniusculus leniusculus* as defined by Sonntag (2006), and clustered in four main clades (Fig. 2). Average pairwise distances among these clades ranged from 2.6% to 3.0%.

Four of the haplotypes detected in Europe were dominant in studied populations: haplotype E3 (in 64 individuals from 23 populations), E7 (99/39), E13 (59/19) and E14 (52/20) (Fig. 1, 2b). In most regions, all four dominant haplotypes together with some rare ones were found, with the exception of Spain where only the common haplotype E14 and one other rarer haplotype was detected, and Finland where only three common haplotypes (E3, E7 and E13) were found (Fig. 1). The remaining 23 haplotypes were less frequent, found in one to 14 individuals in Europe.

Table 1 Summary of sampled *Pacifastacus leniusculus* populations from Europe and North America, their characteristics, number of individuals, and codes of COI haplotypes detected in studied populations. Numbers in parentheses indicate the number of individuals with that particular haplotype. If no number is provided, the same haplotype was detected in all individuals from the respective population.

Country	Locality	Closest settlement	Area	n	Type of waterbody	Date of sampling	Latitude	Longitude	Haplotype codes
Europe									
Region 1									
Great Britain	Teil Burn Five	Teil Burn, Fife	Scotland	7	brook	Jun 2009	56° 13' N	3° 09' W	E7(2), E14(2), E15(3)
Great Britain	River Teith and Row Burn	Blair Drummond, Stirling	Scotland	2	pond and river	Jun 2009	56° 10' N	4° 02' W	E13
Great Britain	Castle Pond	Drumdochty, Auchenblae	Scotland	2	pond	11 Jun 2009	56° 04' N	4° 20' W	E3(1), E7(1)
Great Britain	Bookill Gill Beck	Long Preston	North Yorkshire	6	brook	13 Aug 2009	54° 02' N	2° 14' W	EA6(1), E7(5)
Great Britain	Kennet River	Hungerford	Berkshire	3	river	14 Oct 2009	51° 26' N	1° 31' W	E7(1), E13(1), E14(1)
Great Britain	River Ver	St. Albans	Hertfordshire	8	river	8 Nov 2009	51° 43' N	0° 20' W	E7
Region 2									
Spain	Izal	Izal	Navarra	4	brook	Jul 2004	42° 48' N	1° 08' W	E14
Spain	Arlanzón	Burgos	Castilla y León	9	river	Jun 2008	42° 20' N	3° 42' W	EA6(2), E14(7)
Spain	Duero	Palencia	Castilla y León	4	river	Jul 2008	42° 01' N	4° 32' W	EA6
Region 3									
France	Varenne	Ambrières-les-Vallées	Basse Normandie	4	river	autumn 2008	48° 27' N	0° 39' W	EA6(1), E10(3)
France	Colmont	L'Épinay-le-Comte	Pays de la Loire	4	river	autumn 2008	48° 28' N	0° 49' W	E1(1), E14(3)
France	Aisne	Saint-Aignan-de-Couptrain	Pays de la Loire	2	river	autumn 2008	48° 27' N	0° 20' W	E1
France	Perche	Prélert	Ernée	3	brook	autumn 2008	48° 16' N	0° 51' W	E1(1), EA6(1), E10(1)
France	Meuse	Bassoncourt	Champagne-Ardenne	4	river	8 Oct 2008	48° 04' N	5° 33' E	E5(1), E8(3)
France	Aube	Rouvres-sur-Aube	Champagne-Ardenne	4	river	14 Nov 2008	47° 52' N	4° 59' E	E7(2), E13(2)
France	Ource	Villars-Santenoge	Champagne-Ardenne	4	river	22 Oct 2008	47° 46' N	4° 58' E	E3(1), E7(1), E13(1), E19(1)
France	Petits Crocs	Poinson-lès-Fayl	Champagne-Ardenne	3	river	24 Oct 2008	47° 45' N	5° 34' E	E1(1), E8(2)
France	Chezeau	Roches-Prémarie-Andillé	Vienne	4	brook	27 Jun 2009	46° 29' N	0° 21' E	E3(3), EA6(1)
France	Mesangueville	Dampierre-en-Bray	Seine-Maritime	4	brook	30 Jun 2008	49° 32' N	1° 39' E	E4(1), E18(2), E21(1)
France	Barbuise	Montsuzain	Aube	1	brook	-	48° 27' N	4° 08' E	E1
France	Varenne	-	-	1	-	26 Aug 2008	-	-	E10
France	Rahin	Champagney	Haute-Saône	2	river	-	47° 42' N	6° 41' E	E2(1), E9(1)
France	Hojay	-	-	2	-	26 Aug 2008	-	-	EA6(1), E14(1)
France	Léman	Thonon-les-Bains	Haute-Savoie	1	lake	Jul 2009	46° 23' N	6° 29' E	E7
France	Cherpont	Sainte-Feyre	Creuse	3	brook	4 Jun 2009	46° 09' N	1° 57' E	E3(1), E24(2)
France	Zinzel du Nord	Baerenthal	Moselle	5	brook	3 Sep 2009	48° 59' N	7° 30' E	E13
France	Charpasonne	Panissières	Loire	5	brook	2 Jul 2009	45° 47' N	4° 20' E	E14(2), E27(3)
France	Thoré	St. Amans-Soult	Midi-Pyrénées, Tarn	2	river	1 0ct 2009	43° 29' N	2° 29' E	E26
Italy	Valla	Squaneto	Pareto	6	brook	26 Jul 2009	44° 30' N	8° 22' E	E5

Table 1 (continued)

Country	Locality	Closest settlement	Area	n	Type of waterbody	Date of sampling	Latitude	Longitude	Haplotype codes
Region 4									
Germany	Mümling	Odenwaldkreis	Hessen	4	brook	22 Oct 2009	49° 43' N	9° 00' E	E7(2), E14(1), E16(1)
Germany	Mossaubach	Odenwaldkreis	Hessen	3	brook	28 Oct 2009	49° 38' N	8° 57' E	E7(2), E14(1)
Germany	Nidder	Wetteraukreis	Hessen	4	brook	13 Oct 2009	50° 23' N	9° 07' E	E3(1), E13(3)
Germany	Usa	Hochtaunuskreis	Hessen	4	brook	15 Oct 2009	50° 22' N	8° 37' E	E7(3), E15(1)
Germany	Weil	Hochtaunuskreis	Hessen	4	brook	17 Oct 2009	50° 22' N	8° 24' E	E7(1), E13(2), E22(1)
Germany	Haune	Fulda	Hessen	4	brook	5 Nov 2009	50° 31' N	9° 46' E	E7(3), E13(1)
Germany	Perf	Marburg-Biedenkopf	Hessen	3	brook	18 Oct 2009	50° 53' N	8° 28' E	E13
Germany	Brettach	Hohenlohekreis	Baden-Württemberg	8	river	20 Oct 2009	49° 08' N	9° 31' E	E3(2), E7(3), E14(3)
Netherlands	Oude Leij	Tilburg	Noord-Brabant	7	brook	9 Nov 2009	51° 34' N	4° 59' E	E3(4), E7(3)
Region 5									
Austria	Glan	Feldkirchen	Kärnten	9	river	8 Oct 2008	46° 43' N	14° 07' E	E13(7), E14(2)
Austria	Glan	Feldkirchen	Kärnten	5	river	8 Oct 2008	-	-	E7(1), E13(4)
Austria	Trefling	Mölbling	Kärnten	4	pond	16 Oct 2008	46° 52' N	14° 27' E	E7(3), E14(1)
Austria	Teichbauer	Strassburg	Kärnten	4	pond	16 Oct 2008	46° 54' N	14° 20' E	E7(1), E14(3)
Austria	Wimitzbach	Frauenstein	Kärnten	2	river	13 Aug 2008	46° 47' N	14° 18' E	E1(1), E13(1)
Austria	Gurk	Gurk	Kärnten	9	river	16 Oct 2008	46° 52' N	14° 18' E	E3(6), E7(3)
Croatia	Mura	sv. Martin na Muri	Međimurje	7	river	16 Sep 2009	46° 32' N	16° 23' E	E3(6), E5(1)
Czech Republic	Litomyšl	Jedlová, Litomyšl	East Bohemia	5	pond	15 Apr, 10 Oct 2006	49° 39' N	16° 18' E	E3(2), EA6(1), E7(2)
Czech Republic	Kouba	Sruby	West Bohemia	7	brook	23 May 2006	49° 19' N	13° 01' E	E3(6), E7(1)
Czech Republic	Kozlov	Velké Meziříčí	South Moravia	9	pond	14 Aug, 4 Oct 2006	49° 22' N	16° 04' E	E3(6), E7(3)
Czech Republic	Stržek	Kozlov	South Moravia	5	pond	4 Oct 2006	49° 22' N	16° 04' E	E3(3), E7(2)
Czech Republic	Spustík	Křižanov	South Moravia	6	pond	4 Oct 2006	49° 22' N	16° 07' E	E3(1), E7(5)
Czech Republic	Blanice	Vodňany	South Bohemia	6	river	Sep/Oct 2006	49° 09' N	14° 10' E	E7(5), E23(1)
Hungary	Gyöngyös	Köszeg	Vas	7	river	summer 2005	47° 23' N	16° 32' E	E7(2), E14(5)
Hungary	unnamed pond	Köszeg	Vas	8	pond	summer 2005	47° 23' N	16° 32' E	E7(4), E14(4)
Slovak Republic	Morava	Suchohrad, Záhorie	Bratislava region	2	river	19 Aug 2006	48° 25' N	16° 51' E	E7
Region 6									
Denmark	Alling Å	Randers	Midtjylland	3	brook	Sep/Oct 2008	56° 24' N	10° 09' E	E3(1), E7(1), E12(1)
Denmark	Alling Å	Randers	Midtjylland	5	brook	Sep/Oct 2008	56° 20' N	10° 13' E	E3(3), E7(1), E14(1)
Norway	Ostøya	Sandvika	Akershus	9	pond	29 Sep 2009	59° 52' N	10° 34' E	E1(3), E3(2), E7(1), E13(2), E14(1)
Norway	Brevik	Brevik	Telemark	3	pond	16 Oct 2006	59° 03' N	9° 42' E	E14
Norway	Øymarksjøen	Marker	Østfold	5	lake	23 Jul 2008	59° 19' N	11° 39' E	E3(1), E7(4)

Table 1 (continued)

Country	Locality	Closest settlement	Area	n	Type of waterbody	Date of sampling	Latitude	Longitude	Haplotype codes
Region 6 (continued)									
Sweden	Träsksjön	Karlsudd	Stockholms Län	7	lake	-	59° 24' N	18° 12' E	E1(3), E14(2), E16(1), E20(1)
Sweden	Skillötsjön	Gnesta (Södertälje)	Stockholms Län	6	lake	Aug 2006	59° 03' N	17° 22' E	E3(3), E11(3)
Sweden	Mjögan	Arvika	Värmlands Län	5	river	10 Jan 2007	59° 58' N	12° 30' E	E3(1), E7(1), E13(3)
Sweden	Högsäterälven	Eda	Värmlands Län	3	river	27 Jul 2005	59° 52' N	12° 23' E	E7
Sweden	Stora Le	Dals-Ed	Värmlands Län	2	lake	13 Sep 2006	59° 02' N	11° 52' E	E13(1), EA25(1)
Sweden	Fagerhultasjön	Småland	Jönköpings Län	5	lake	19 Sep 2008	57° 30' N	15° 20' E	E13
Region 7									
Latvia	Salaca River	Salacrgīva	Limbažu rajons	10	river	Sep 2009	57° 45' N	24° 22' E	E7(1), E13(9)
Poland	Pobłędzie	Skajzgiry	Warmińsko-Mazurskie	10	lake	31 Aug 2009	54° 18' N	22° 45' E	E1(1), E3(1), E7(2), E14(5), E18(1)
Poland	Naryjska Struga	Morag	Warmińsko-Mazurskie	10	river	17 Aug 2009	53° 56' N	19° 59' E	E3(4), E7(5), E17(1)
Region 8									
Finland	Saimaa	Rantatie	Etelä-Karjala	10	lake	Aug/Sep 2009	61° 17' N	27° 59' E	E7(6), E13(4)
Finland	Kukkia	Luopioinen	Pirkanmaa	10	lake	13 Jul 2004	61° 22' N	24° 40' E	E3(5), E7(2), E13(3)
North America									
USA									
Montana	Bitterroot River	Hamilton	Ravalli Co.	5	river	31 Aug 2010	46° 17' N	114° 10' W	EA6
Montana	Blackfoot River	Bonner	Missoula Co.	5	river	6 Sep 2010	46° 53' N	113° 50' W	EA6
Washington	Snake River	Wawawai Bay	Garfield Co.	9	river	May 2009	46° 38' N	117° 23' W	EA6(1), EA25(7), A34(1)
Washington	Chehalis River	Porter	Grays Harbor Co.	6	river	8 Oct 2010	46° 56' N	123° 19' W	A36(1), A37(2), A38(1), A39(2)
Oregon	North Fork Clackamas River	Portland	Clackamas Co.	6	river	Oct 2009	45° 15' N	122° 17' W	A28(1), A29(1), A30(1), A31(1), A32(1), A33(1)
Canada									
British Columbia	Goldstream River	Vancouver Island		1	river	-	48° 29' N	123° 33' W	A35

Crayfish from Lake Träsksjön, Sweden, which received the first small batch of *P. leniusculus* imported from California, differed in their haplotype composition from other Swedish localities (with which it did not share any haplotype) but did stand out when compared with the overall variation in Europe. Two out of seven analysed individuals from this lake carried the common haplotype E14, two other detected haplotypes (E1 and E16) belonged to relatively rare ones, and one haplotype (E20) was unique for this locality.

Fourteen haplotypes were detected in 32 signal crayfish from five North American populations. Most of these fell within *Pacifastacus l. leniusculus*. Twelve were present only in American samples (A28-A39) but two were also detected in Europe (EA6 in several countries, EA25 in one Swedish population). Four haplotypes (A36-A39) detected in six samples from the Chehalis River (Washington) represent a new, highly divergent lineage within *P. leniusculus* (Fig. 2a). Its average COI divergence from *P. l. leniusculus* is 7.0%, the divergence from *P. l. klamathensis* is 6.8% and from *P. l. trowbridgii* 6.2%.

Discussion

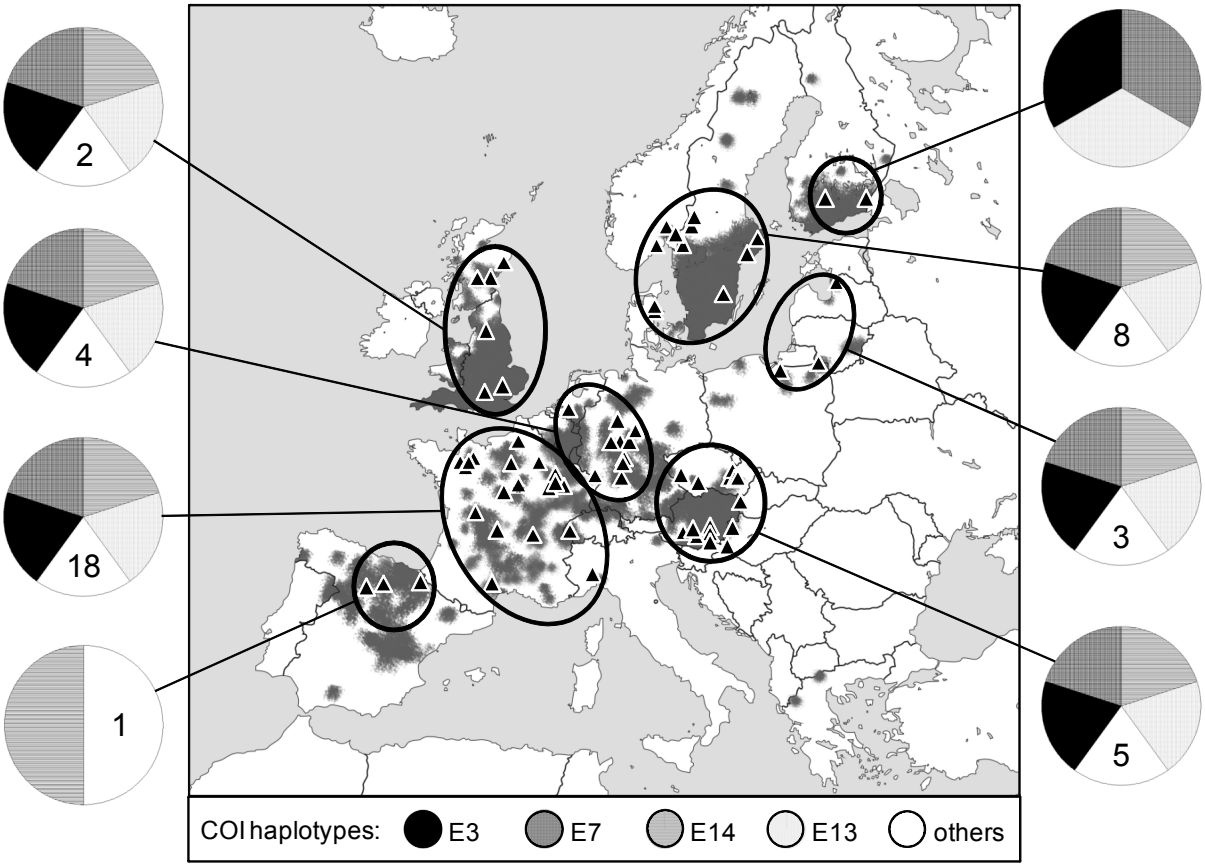
This first large-scale study of genetic variation in the signal crayfish *Pacifastacus leniusculus* in Europe clarified the subspecies status of populations in this introduced European range; however, our results from a limited number of American populations at the same time challenge the present view on the diversity of this species in North America.

Based on literature data (Agerberg and Jansson 1995, Grandjean and Souty-Grosset 1997, Hobbs 1989) and on the fact that at least two subspecies are present in the American source locality for numerous signal crayfish introduced to Europe, presence of more subspecies of *Pacifastacus leniusculus* was expected on the continent. However, we only found individuals carrying haplotypes related to *Pacifastacus leniusculus leniusculus* (as proposed by Sonntag 2006) in the analysed European populations. We cannot exclude the presence of another lineage in some restricted unsampled area in Europe, but it is obvious that *P. l. leniusculus* is largely dominant on the continent.

Despite belonging to a single major lineage, high genetic variation was found in European populations of *P. leniusculus leniusculus*. Values of average pairwise divergences found between four major clades of haplotypes detected in Europe were relatively high, reaching up to 3.0%. In the family Astacidae, into which signal crayfish belongs, these levels correspond to intraspecific divergence (Trontelj et al. 2004); however, in another family of American crayfish, Cambaridae, such divergences are frequently observed among different species (Taylor and Knouft 2006). In another group that includes a successful invader, the *Orconectes virilis* species complex, average pairwise divergences (Kimura-2-parameter)

among different lineages, that are considered recently diverged cryptic species, range from 1.2% to 4.1% (Filipová et al. 2010, Mathews et al. 2008).

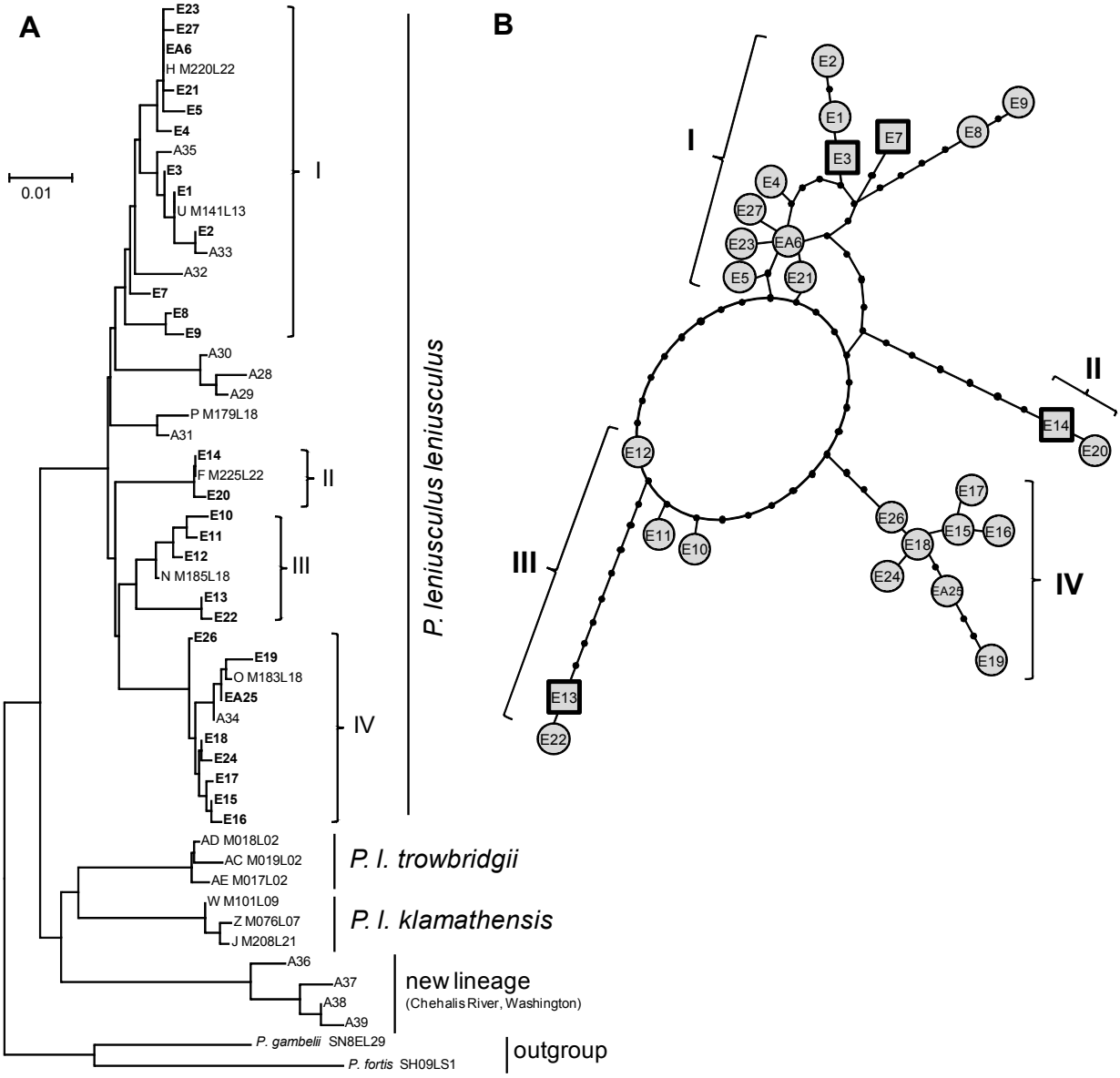
Fig. 1 Distribution of *Pacifastacus leniusculus* in Europe (compiled from Candiotto et al. 2010, Capurro et al. 2007, Holdich et al. 2009, Souty-Grosset et al. 2006, Vrålstad et al. 2011 and **chapter VI**) with localities sampled in our study (black triangles). Pie charts show the presence of four dominant COI haplotypes (E3, E7, E14, E13) and less frequent haplotypes in main regions in Europe. Numbers in white sections of the pie charts indicate the number of other (rare) haplotypes detected in the respective regions.



The high variation found within signal crayfish in Europe is in contrast with another widespread invader, the spiny-cheek crayfish *Orconectes limosus*. In this species, a single introduction of 90 individuals from North America resulted in a successful colonisation of a large part of the European continent but the introduction bottleneck led to very low level of mtDNA variation (one dominant, and one very rare COI haplotype) in its European populations (Filipová et al. 2011b). In the third most widespread invasive crayfish in Europe, the red swamp crayfish *Procambarus clarkii* that was introduced to the continent in 1973 by about 40 000 individuals from Louisiana (USA), analyses of mitochondrial and nuclear markers showed intermediate levels of variation (Barbaresi et al. 2007). Five COI haplotypes

detected in Europe were closely related, reflecting recent colonization of *P. clarkii* in Europe. Geographic patterns of variation revealed by microsatellite markers corresponded to a scenario of multiple secondary introductions of individuals across Europe, originating in different source localities.

Fig. 2 Neighbour-joining tree (A) showing the COI sequence variation of signal crayfish. The tree comprises all haplotypes found in studied European and American populations, and representative sequences of the three presently recognised signal crayfish subspecies (from Sonntag 2006). Geographical distribution of detected haplotypes is reported in Table 1. Haplotype network (B) of European COI haplotypes, gathering into four main clades (labelled I, II, III and IV). Four common haplotypes (E3, E7, E13 and E14) are marked by squares.



In our study, we did not observe any clear geographical pattern in the distribution of haplotypes detected in European signal crayfish. This corresponds to the history of colonization of Europe by this species, which was introduced in large numbers and it was then distributed around the continent by numerous secondary human-mediated translocations. However, in some of the studied regions the haplotype composition seemed related to the local history of introduction of the species. In the Czech Republic, for example, E3 and E7 haplotypes were largely dominant, while the other two haplotypes common across Europe, E13 and E14, were absent (Table 1). This might also be due to the introduction bottleneck. All Czech populations are most likely descendants from a single batch of 1000 juvenile individuals imported in 1980, which were released to a few isolated localities in the country (Ircing and Prášil 1980, Policar and Kozák 2000). However, most of the initial releases were unsuccessful, so the number of founders from which other Czech localities were stocked was substantially lower (Kozák et al. 1998). Interestingly, for many years after introduction, the distribution of the species seemed to remain restricted in the country, and only recently it has been observed that the species expands its range (Nature Conservation Agency of the Czech Republic, unpublished data).

Low numbers of haplotypes detected in the three localities in northeastern Spain (one dominant and one rare) and in the single analysed population in northern Italy (rare haplotype E5; Table 1), may reflect relatively recent introductions of signal crayfish to these regions (Candiotto et al. 2010, Diéguez-Uribeondo 2006, Oscoz et al. 2010). However, more extensive sampling would be needed to confirm that founder events indeed substantially reduced the genetic variation of signal crayfish in these areas. For detailed studies of *P. leniusculus* invasions in Europe, analyses of more variable markers such as microsatellites might be useful, as was shown e.g., in *Procambarus clarkii*, where microsatellite and also RAPD markers were used to investigate the extent and patterns of genetic variation in its European populations (Barbaresi et al. 2003, 2007).

Analysis of additional North American populations revealed that although most individuals were genetically related to *Pacifastacus leniusculus leniusculus* and none fell within the lineages attributed to by Sonntag (2006) to *P. l. klamathensis* and *P. l. trowbridgii*, a new highly divergent lineage was found in the Chehalis River, Washington. This shows that genetic diversity within *P. leniusculus* is higher than currently recognised, which corresponds to findings of E.R. Larson (pers. comm.) who has recently discovered four distinct lineages (16S mtDNA) within signal crayfish from the Pacific Northwest in North America (one of them, apparently corresponding to the same clade, distributed in the Chehalis River).

The real taxonomic status of this clade, as well as of the three presently recognised signal crayfish subspecies, is also unclear. Genetic divergences among the three lineages detected by Sonntag (2006) are high, corresponding rather to values found between different

species, with average pairwise divergence (Kimura 2-parameter distance) around 4-5% (L. Filipová, unpubl. data). Based on Sonntag's results, Schuster et al. (2010) already noted that the three subspecies might represent distinct biological species. Further sampling from North America and detailed studies of biology, distribution and reproduction of these cryptic groups is therefore needed to understand their relationship, diversity, and conservation value.

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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.

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INVASION NOTE

Cryptic diversity within the invasive virile crayfish *Orconectes virilis* (Hagen, 1870) species complex: new lineages recorded in both native and introduced ranges

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Abstract The virile crayfish (*Orconectes virilis*) represents a cryptic species complex with several lineages known in the USA, and a wide introduced range. In Europe, *O. virilis* is an emerging invader, established during the last decade in at least two areas—one in the United Kingdom and another in the Netherlands. We assessed the position of both known European populations within the species complex by sequencing part of the mitochondrial gene for cytochrome *c* oxidase subunit I. Tested UK and Dutch individuals did not belong to any mitochondrial lineage recorded in North America so far but formed a separate clade, the original distribution area of which is unknown. Additionally sequenced virile crayfish from Iowa (USA) also represented a new clade, suggesting that undiscovered lineage variation within *O. virilis* remains high. This exemplifies that genetic analyses of invading populations may provide new insights into diversity of a taxon in its original range.

Keywords Invasive crayfish · Cryptic diversity · North America · Europe · COI

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Introduction

Several North American crayfish species have been introduced to Europe since 1890 to substitute for lost populations of indigenous crayfish, decimated by the pathogen of the crayfish plague, oomycete *Aphanomyces astaci* (Souty-Grosset et al. 2006). Three of these non-indigenous crayfish have spread over a large part of the European continent: the spiny-cheek crayfish *Orconectes limosus* (Rafinesque, 1817), the signal crayfish *Pacifastacus leniusculus* (Dana, 1852), and the red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Henttonen and Huner 1999). Besides the ability of these invasive species to compete with the indigenous European crayfish, all of them can also transmit crayfish plague and cause mass mortalities of the indigenous species (Souty-Grosset et al. 2006). The negative effect of North American crayfishes on indigenous species is well-documented (Holdich 1999; Lodge et al. 2000; Souty-Grosset et al. 2006). Several other North American crayfish species have been recorded in European surface waters during the last few years (Holdich and

Pöckl 2007), probably introduced through the aquarium trade. These include the calico crayfish *Orconectes immunis* (Hagen, 1870), present in southwest Germany since 1997, the marbled crayfish ("Marmorkrebs", *Procambarus* sp.) found in 2003 in the wild in Germany and in 2004 in the Netherlands, and *Orconectes juvenilis* (Hagen, 1870), first recorded in 2005 in France (Chucholl and Daudey 2008).

Another North American species thought to be introduced to Europe through this pathway is the virile crayfish, *Orconectes virilis* (Hagen, 1870). Its distribution in North America is very wide (Hamr 2002), ranging from southern Canada in the north to Texas in the south, and from Utah and Montana in the west to New York in the east (Fig. 1). On a large part of its North American territory, the virile crayfish is nevertheless considered non-indigenous (Fig. 1), including some parts of Canada (McAlpine et al. 1999, 2007), Chihuahua and Baja California Norte in Mexico, US Southwest and parts of the US Atlantic coast (Hobbs 1989; Hamr 2002). Similarly to other invasive cambarid crayfishes, high abundances of *O. virilis* specimens may have detrimental effects on their environment, such as a negative impact on submerged macrophytes and increase of water turbidity (Souty-Grosset et al. 2006; Soes 2007).

Recent data have shown that populations identified in the USA as the virile crayfish represent, together with additional related species, a highly diversified cryptic species complex (Mathews et al. 2008). Apart from previously recognised taxa *O. deanae* and *O. nais*, at least four divergent lineages exist within *O. virilis* sensu lato, well separated at mitochondrial (16S rRNA, cytochrom *c* oxidase subunit I) as well as nuclear (glyceraldehyde-3-prosphate dehydrogenase) markers. At least three of them also exhibit morphological differences (Mathews et al. 2008), and one of them (labelled as the lineage 2 in the above-mentioned paper) has already been described as *Orconectes quinebaugensis* Mathews & Warren, 2008. However, samples of the virile crayfish analysed in Mathews et al. (2008) originated from a relatively small area within the entire range of the taxon distribution in North America

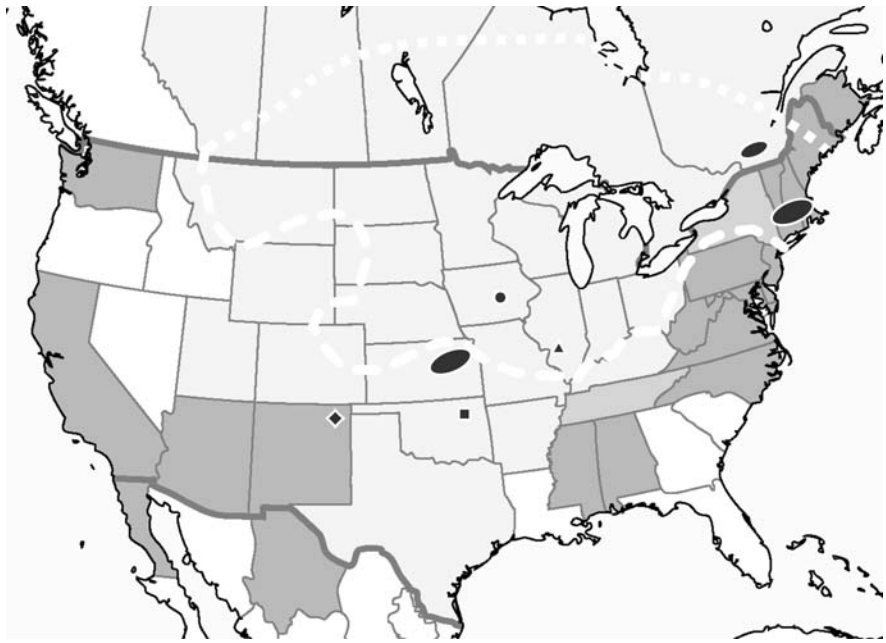

Fig. 1 Distribution and status of *Orconectes virilis* (sensu lato) in administrative units within its present range in North America (compiled from Global Invasive Species Database: www.invasivespecies.net; Hobbs 1989; Hamr 2002; Taylor et al. 2007; McAlpine et al. 1999, 2007). *Shading* indicates the presence and status (*pale*: native, *dark*: introduced; *intermediate*: status unclear) of *O.* cf. *virilis* in the particular region, not its exact distribution. *White dashed line* shows the presumed original range (northern limits indicated as unclear) according to Page (1985). Sources of samples for the present and previous studies are indicated by *black symbols* as follows: Mathews et al. (2008): *ovals—O.* cf. *virilis*, *diamond—O. deanae*, *square—O. nais*; Taylor and Hardman (2002): *triangle*; our study (Iowa): *circle*

(Fig. 1). It might therefore be expected that even more distinct lineages exist within this complex. Allozyme data on populations from several localities in the USA (Indiana, Illinois and Missouri), different from those studied by Mathews et al. (2008), also suggest that reproductive isolation among geographically distant populations of the virile crayfish is common (Fetzner et al. 1997).

At least two unsuccessful attempts to introduce *O. virilis* to Europe were made in the past. First the crayfish were released in France in 1897 (Arrignon et al. 1999) and later, in 1960, into Swedish waters (Skurdal et al. 1999). The first established European population of the virile crayfish was recorded in 2004 in the Netherlands at Vinkeveen near Amsterdam from where it has been quickly spreading to neighbouring waters (Souty-Grosset et al. 2006; Soes 2007). This population is supposed to come from the aquarium trade and it has apparently already been present in the area several years before (Pöckl et al. 2006). Virile crayfish have already colonised several hundred kilometres of Dutch waterways, and are likely to spread quickly further (Soes 2007). The species will probably significantly influence the ecology of Dutch waters (Souty-Grosset et al. 2006).

Around the same time as in the Netherlands, in 2004, a population of non-indigenous crayfish was discovered within the River Lee system of North London in the United Kingdom (Ahern et al. 2008). First believed to be the spiny-cheek crayfish, *Orconectes limosus*, they were identified as *O. virilis* after a detailed re-examination of their morphology by D. Ahern and one of authors (DMH) and subsequently confirmed by C. A. Taylor. The possible source of this population seems to be the contents of an aquarium tank tipped into a pond in north London by a local resident (Ahern et al. 2008). However, the exact origin of the population remains unknown.

In this study, we sequenced the mitochondrial gene for the cytochrome *c* oxidase subunit I (COI) of selected *O. virilis* individuals from British and Dutch populations to assess their position within the complex, and to test whether they are closely related or originate from clearly different sources, which would indicate independent invasions. Additionally, we included in the analysis an individual sampled in Iowa (USA), outside the regions from which virile crayfish have been studied genetically, to further evaluate the extent of cryptic diversity of the virile crayfish species complex.

Materials and methods

Three individuals of *Orconectes* cf. *virilis* from the River Lee system in north London (UK; 51°36′N, 0°2′W) were obtained from the Environment Agency. Two virile crayfish were sampled near Kanis close to Woerden (the Netherlands; 52°8′N, 4°53′E). An additional male individual was collected in Squaw Creek near Cedar Rapids in Iowa (USA; 41°58′N, 91°40′W). Sampled material was preserved in ethanol.

One segment of leg of each captured crayfish was dissected to obtain muscle tissue, from which the genomic DNA was subsequently extracted using the Chelex extraction: ~ 1 mm^3 of the muscle tissue was placed in a solution of 175 µl of distilled water and 5 µl of proteinase K (20 mg/ml), and homogenised; subsequently, 175 µl of H_2O were added and the contents were homogenised again. Finally, a small amount (about 50 µl) of Chelex 100 beads was added, the Eppendorf tubes were vortexed gently and incubated at 56°C for 4 h, followed by incubation at 100°C for 8 min to denature the proteins. Chelex resin and undigested solids were removed from the suspension by centrifugation for 4 min at 12,000 rpm, and the supernatant was stored at −20°C.

PCR reaction mixtures of the volume 25 µl contained 5× PCR Buffer (1.5 mM $MgCl_2$) (Promega), 200 µM dNTP, 25 µM each primer, 0.625 units Taq polymerase (Promega), 0.5 µl of the template (about 100 ng DNA). Primers HCO 2198 and LCO 1490 (Folmer et al. 1994) were used to amplify the COI gene fragment. The amplification program consisted of an initial denaturation step of 5 min at 95°C, 35 cycles of 50 s at 95°C, 50 s at 55°C and 50 s at 72°C, and a final extension for 5 min at 72°C. PCR products were purified using Exonuclease I (New England Biolabs) and Shrimp Alcaline Phosphatase (Fermentas), with incubation for 1 h at 37°C followed by 20 min at 80°C. Purified products were then sequenced using the LCO primer and BigDye v. 3.1 Terminator kit on a capillary sequencer (ABI PRISM 3130). Resulting sequences were deposited in GenBank (accession numbers FJ608575-FJ608578).

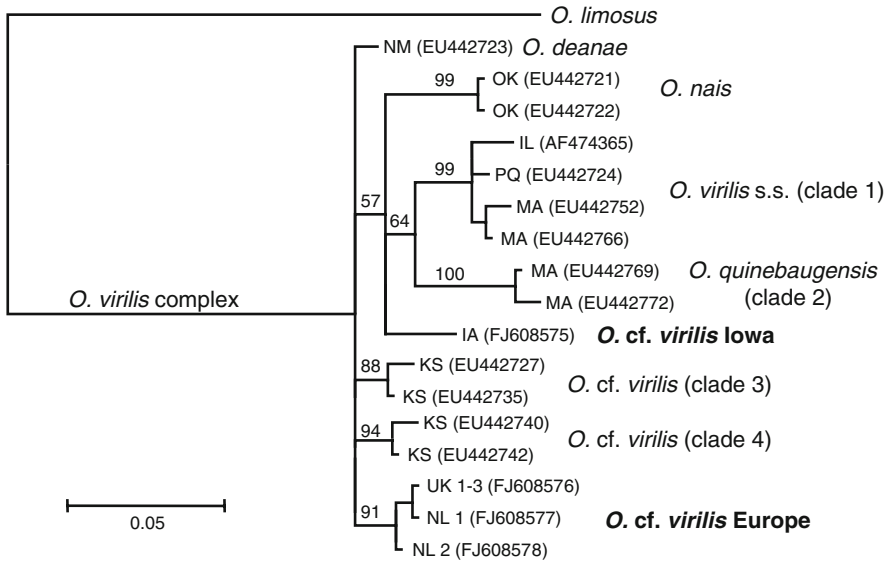

Fig. 2 Diversity of known lineages of the *Orconectes virilis* species complex from North America and Europe. The tree was created by the Bayesian inference of phylogeny from 486 bp long alignment of the partial mitochondrial gene for the cytochrome *c* oxidase subunit I. *Numbers* at selected nodes indicate the percentage of sampled trees containing that particular node, those with support below 50% were collapsed to polytomy. GenBank accession numbers of individual sequences are listed in *parentheses*. Clades within the complex are labelled after Mathews et al. (2008) and Mathews and Warren (2008), newly analysed lineages are marked by *bold font*. State and country abbreviations: IA—Iowa, IL—Illinois, KS—Kansas, MA—Massachusetts, NL—Netherlands, NM—New Mexico, OK—Oklahoma, PQ—Quebec, UK—United Kingdom. *Scale* represents 5% divergence

COI sequences representing divergent haplotypes and various geographic regions from all known lineages of the *O. virilis* complex (2–4 per clade), including *O. quinebaugensis*, *O. nais* and *O. deanae*, analysed previously by Mathews et al. (2008), and *O. virilis* from Illinois analysed by Taylor and Hardman (2002), were obtained from GenBank. Accession numbers of these sequences are provided in Fig. 2. A COI sequence of *Orconectes limosus* (GenBank acc. no. EU442747) was used as an outgroup in subsequent analyses. Sequences were aligned in the software Mega 4.0 (Tamura et al. 2007), and truncated to a length available for all individuals (486 bp). In the same program, we calculated the average pairwise divergence between different clades of the species complex using the Kimura 2-parameter model.

The tree showing the diversity of the *O. virilis* complex including the British, Dutch and Iowa individuals was constructed using the Bayesian inference of phylogeny in MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003), under a HKY+I model (Hasegawa et al. 1985), determined by Akaike information criterion in Modeltest version 3.7 (Posada and Crandall 1998). We used two parallel runs of four Monte Carlo Markov chains, each run for 3 million generations and sampling every 100 generations; the initial 20% of trees were discarded as a burn-in phase. Analyses based on alternative models of evolution well-scoring in Modeltest (HKY+G, GTR+G) showed identical patterns.

Results

The position of the newly analysed samples within the *O. virilis* complex is shown in Fig. 2. All tested European individuals of *O.* cf. *virilis* belonged to the same lineage, divergent from any clade so-far known from the North American range of the complex. Three crayfish from the London population and one from the Netherlands shared the same haplotype; the haplotype of the second Dutch individual differed by a single point mutation. The average COI divergence (Kimura 2-parameter distance) of the "European" lineage from other clades of the complex ranged from 1.21% (to *O. deanae*) to 3.90% (to *O. quinebaugensis*). These values of divergence, despite being relatively

Table 1 Average pairwise divergences (Kimura 2-parameter model) between different clades of the *Orconectes virilis* species complex (named as in Fig. 2)

	O. virilis complex						
	Europe (%)	Iowa (%)	Clade 1 (%)	Clade 2 (%)	Clade 3 (%)	Clade 4 (%)	*O. deanae* (%)
Europe	–						
Iowa	2.93	–					
O. virilis s.s. (clade 1)	3.31	2.91	–				
O. quinebaugensis (2)	3.90	3.85	3.13	–			
O. cf. *virilis* (clade 3)	1.64	2.54	2.43	3.41	–		
O. cf. *virilis* (clade 4)	1.85	2.32	3.14	4.08	1.47	–	
O. deanae	1.21	2.11	2.48	3.41	1.26	1.47	–
O. nais	2.71	3.18	3.57	4.06	3.19	3.41	2.32

low, are comparable to divergence between other lineages within the complex (Table 1): for example, the divergence between *O. deanae* and clade 3 of *O.* cf. *virilis* was only 1.26%.

The sample from Iowa represented an apparently new lineage of the complex as well; its average divergence from other clades of the complex varied between 2.11% (to *O. deanae*) and 3.85% (to *O. quinebaugensis*).

The average pairwise divergence among all clades of the *O. virilis* complex sampled in North America (including the virile crayfish from Iowa, *O. nais* and *O. deanae*), was 2.85%; their average divergence to the European lineage was 2.51%.

Discussion

Individuals of *O. virilis* from Europe and Iowa analysed in this study represent new lineages of the *O. virilis* complex, substantially divergent from other clades of the species complex known from the examined part of its range in North America. Our results confirm that both European populations of *O.* cf. *virilis*, the Dutch and British one, belong to the same lineage, the native distribution of which remains unknown, as no information is available on the origin of founders of these populations. It is possible that both localities were stocked by crayfish originating from the same wild source and subsequently spread through aquarium trade. More information about variation of the *O. virilis* complex in other parts of its North American range, and about the availability of these crayfish on the hobbyist market, is necessary to identify the source region of the "European" lineage in North America.

"European" virile crayfish, as well as two other American lineages of the complex (clade 2 and 3), are apparently genetically very close to *O. deanae*. However, the latter species is considered distinct based on its morphological features, despite its genetic similarity to other American clades of the *O. virilis* complex (the average Kimura 2-parameter distance 2.36%, even lower than the divergence of the new "European" lineage from other American ones).

The average COI divergences among all currently known members of the species complex, including *O. nais* and *O. deanae*, did not exceed 3%. In a number of animal groups, such levels of divergences could represent intraspecific variation (Hebert et al. 2003). Costa et al. (2007) actually showed that among crustaceans, the average divergence of congeneric species was about 17% (Kimura 2-parameter distance), the highest value detected so far in animals. However, divergences between many *Orconectes* species are much lower. Taylor and Knouft (2006) provided sequences of 86 species or subspecies of the genus. The divergences between several sister pairs of well-described species used in their study were variable. The lowest value, between *O. peruncus* and *O. quadruncus,* was 3.3%, confirming that many currently recognised *Orconectes* species are very closely related.

Two European species of another crayfish genus, *Austropotamobius pallipes* and *A. torrentium*, both

showed deep phylogeographic structures (Trontelj et al. 2005), with COI divergences between major geographic clades (uncorrected values 5.9 and 4.1%, respectively) substantially exceeding values observed among lineages within the *O. virilis* complex. However, the existence of differences in morphology of some *O. virilis* clades (Mathews et al. 2008), differences between *virilis* lineages detected at nuclear markers (Fetzner et al. 1997; Mathews et al. 2008), as well as a serological distinctness of the morphologically very close species *O. nais* (Pryor and Leone 1952), suggest that the virile crayfish complex is young, recently diversifying and already consists of a number of distinct biological species. Mathews et al. (2008) propose that the complex has undergone radiation since the late Pleistocene, with the divergences among clades originating within the last 2 million years. Such diversification may be a result of a substantial range fragmentation during Pleistocene glaciations.

Our finding of a new member of the species complex in invasive populations outside its native range is not the only case of a discovery of unknown crayfish distributed through the aquarium trade. The most infamous is another cambarid species, the parthenogenetically-reproducing marbled crayfish, which is widespread among aquarists but not known from North American waters (Scholtz et al. 2003). It has been already found in the wild in Europe (Souty-Grosset et al. 2006), and represents a potentially serious threat to local aquatic biota. Our results are a good example of a case where genetic analyses of invaders' populations improve knowledge about biodiversity of the taxon in its original distribution areas.

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CHAPTER V

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Identification of exotic North American crayfish in Europe by DNA barcoding

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ABSTRACT

Key-words: *COI, barcoding, invasive crayfish, Europe, North America,* Orconectes, Procambarus

Several alien crayfish of North American origin have become established in Europe in recent decades, but their identification is often confusing. Our aim was to verify the taxonomic status of their European populations by DNA barcoding. We sequenced the cytochrome *c* oxidase subunit I (COI) gene fragment of individuals representing all American crayfish known from European waters, and compared the results with reference sequences from North America. Our results confirm the morphological identification of *Orconectes juvenilis* from a population in eastern France, and of the marbled crayfish (Marmorkrebs), *i.e.*, a parthenogenetic form of *Procambarus fallax*, from south-western Germany. Sequences of most individuals of presumed *Procambarus acutus* from the Netherlands were similar to American *P*. cf. *acutus*, but one was divergent, closer to a sequence of a reference individual of *P*. cf. *zonangulus*. However, divergences among three American *P*. cf. *zonangulus* samples were also high, comparable to interspecific variation within cambarid species complexes. The divergence between *O. immunis* from Europe and America also reached values corresponding to those observed among distinct *Orconectes* species. Genetic variation in the American range of these crayfish should therefore be further studied. Our study shows that DNA barcoding is useful for the rapid and accurate identification of exotic crayfish in Europe, and also provides insights into overall variation within these taxa.

RÉSUMÉ

Identification des écrevisses d'Amérique du Nord introduites en Europe par une méthode de DNA barcoding (code-barre génétique)

Mots-clés : *COI, code-barre génétique,*

Plusieurs écrevisses provenant d'Amérique du Nord se sont établies en Europe pendant ces dernières dizaines d'années, mais leur identification est encore souvent confuse. Notre but était de vérifier le statut taxonomique de leurs populations en Europe en utilisant une méthode de DNA barcoding (code-barre génétique).

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écrevisses invasives, Europe, Amérique du Nord, Orconectes, Procambarus

Nous avons séquencé le gène codant pour la sous-unité I de la cytochrome *c* oxydase (COI) chez des individus qui représentent l'ensemble des écrevisses américaines rencontrées dans les eaux européennes. Ces données ont été ensuite comparées avec les séquences de référence d'individus d'Amérique du Nord. Nos résultats confirment l'identification morphologique d'*Orconectes juvenilis* provenant d'une population de l'est de la France et des écrevisses marbrées (Marmorkrebs), correspondant à la forme parthénogénétique de *Procambarus fallax*, du sud-ouest de l'Allemagne. Les séquences de la plupart des individus des Pays-Bas supposés appartenir à *Procambarus acutus* sont similaires à *P*. cf. *acutus* d'Amérique. Cependant, une séquence s'est révélée différente des autres, étant plus proche de séquence d'un individu de référence de *P*. cf. *zonangulus*. Néanmoins, les divergences parmi trois échantillons américains de *P*. cf. *zonangulus* sont aussi élevées et comparables à la variation interspécifique au sein des complexes d'espèces des Cambaridae. Les divergences entre *O. immunis* d'Europe et d'Amérique ont également atteint des valeurs similaires à celles qui ont été observées parmi des espèces d'*Orconectes* distinctes. La variation génétique dans l'aire de répartition de ces écrevisses en Amérique du Nord devrait donc être étudiée en détail. Ainsi, notre étude montre que la méthode de DNA barcoding est utile pour l'identification rapide et précise des écrevisses introduites en Europe. Elle peut également apporter de plus amples connaissances sur la variation totale dans ces taxa.

INTRODUCTION

About 120 years ago, the first crayfish originating from a different continent, the North American spiny-cheek crayfish *Orconectes limosus* (Rafinesque, 1817), became successfully established in Europe (Kossakowski, 1966). This was later followed during the 20th century by intentional introductions of several other exotic crayfish species (Gherardi and Holdich, 1999). Three of these became very widespread: the firstly introduced *O. limosus*, plus the signal crayfish *Pacifastacus leniusculus* (Dana, 1852) and the red swamp crayfish *Procambarus clarkii* (Girard, 1852). Given the importance of these invasive species for colonized aquatic ecosystems and for conservation of native crayfish species, their ecology, distribution, and impact have been studied intensively in recent decades (Gherardi and Holdich, 1999; Souty-Grosset *et al.*, 2006). The genetic variation of European populations of these invaders has also received some attention (Grandjean and Souty-Grosset, 1997; Barbaresi *et al.*, 2007; Filipová *et al.*, 2009; Hulák *et al.*, 2010). Recently, established populations of several other North American crayfish have been discovered in European waters. Most of these species, introduced to the wild after the mid-1990s, are likely to have come from the aquarium trade and, in contrast to well-established non-indigenous crayfish species ("Old NICS"), they are sometimes referred to as "New NICS" (Holdich *et al.*, 2009).

The oldest "New NICS" of North American origin in Europe, the calico crayfish *Orconectes immunis* (Hagen, 1870), was first recorded in the mid-1990s in southern Germany (Dussling and Hoffmann, 1998; Gelmar *et al.*, 2006). At present, it colonizes a stretch of approximately 100 km of the Upper Rhine plain in south-western Germany and eastern France (Holdich, 2003; Chucholl, 2006; Chucholl *et al.*, 2008; M. Collas, pers. comm.). Its taxonomical status has never been questioned; however, the identification of other recently established American invaders has not been so straightforward.

A crayfish population found in 2004 in London, Great Britain, was first considered to be *Orconectes limosus*, but subsequent morphological examination of local individuals resulted in re-identification as the virile crayfish *Orconectes virilis* (Hagen, 1870) (Ahern *et al.*, 2008). Similarly, *O. virilis* was known to be established in the Netherlands at least since 2004 (Soes and van Eekelen, 2006). Genetic analysis of individuals from both these European populations nevertheless revealed that they represent a new lineage of the *O. virilis* species complex; its original distribution in North America is not yet known (Filipová *et al.*, 2010).

In 2005, a population of another *Orconectes* species was found in the watershed of the river Doubs in eastern France (Daudey, 2006; Chucholl and Daudey, 2008). These crayfish were first identified as the highly invasive rusty crayfish *Orconectes rusticus* (Girard, 1852) (Daudey, 2006), and this identification continued to be used by authors of influential reference publications (Pöckl *et al.*, 2006; Souty-Grosset *et al.*, 2006). However, Chucholl and Daudey (2008) subsequently examined the morphology of these individuals in detail and identified them as *Orconectes juvenilis* (Hagen, 1870), as was also confirmed by Christopher A. Taylor (Illinois Natural History Survey). This species is relatively closely related to but distinct from *O. rusticus* (Taylor, 2000).

The identification of recently established species of the genus *Procambarus* in Europe is no less problematic. A good example is the complicated taxonomic history of the marbled crayfish ("Marmorkrebs"). This unusual lineage, the only known obligately parthenogenetic crayfish, started to spread in the German and Austrian aquarium trade in the mid-1990s (Scholtz *et al.*, 2003; Souty-Grosset *et al.*, 2006). It has received substantial research attention since the early 2000s (Scholtz *et al.*, 2003) but was only recently unambiguously confirmed to be an asexual form of *Procambarus fallax* (Hagen, 1870) by both morphological and molecular approaches (Martin *et al.*, 2010a). Marbled crayfish have been found in the wild in the Netherlands, Germany and Italy (Soes and van Eekelen, 2006; Souty-Grosset *et al.*, 2006; Marzano *et al.*, 2009; Chucholl and Pfeiffer, 2010; Martin *et al.*, 2010b), although it seems that a well-established reproducing population has so far only been confirmed from south-western Germany (Chucholl and Pfeiffer, 2010).

Since at least 2005, another *Procambarus* species has been spreading in the Netherlands (Soes and van Eekelen, 2006). Its identity has not yet been unambiguously confirmed; it is considered to probably be *P. acutus* (Girard, 1852), but identification as *P. zonangulus* Hobbs and Hobbs, 1990 has also been discussed (Holdich *et al.*, 2009). The presence of *P. zonangulus* in the wild in Germany was suggested by several authors (Dehus *et al.*, 1999; Westman, 2002), but this was not later confirmed (Holdich, 2003). The taxonomy of these species, both generally referred to as white river crayfish, is not clear even in North America; with at least one closely related species being still undescribed (Huner *et al.*, 1994; Fetzner *et al.*, 1997).

The history of most "New NICS" of North American origin given above clearly shows that identification of such invaders is not a simple task. To obtain reliable conclusions, one should not rely exclusively on a single approach (*e.g.*, morphological examination of a few individuals) but more complex analyses are recommended. Classical approaches may be well combined with the analysis of species-specific molecular markers, which now allows relatively simple testing of taxonomic hypotheses. DNA barcoding, *i.e.*, the use of standardized DNA sequences for species identification, was proposed in the early-2000s as a particularly promising tool (Hebert *et al.*, 2003). Although any single molecular marker should be used with care, especially as some publicly available reference data may be flawed (Buhay, 2009) or from misidentified specimens, mitochondrial DNA sequences have turned out to be a suitable method for crayfish identification. In addition to the above-mentioned cases (Filipová *et al.*, 2010; Martin *et al.*, 2010a), this approach has been successfully used, for example, to confirm the identity of marbled crayfish found in Italy (Marzano *et al.*, 2009) and Germany (Martin *et al.*, 2010b), or of *Cherax destructor* (Clark, 1936) recently established in Italy (Scalici *et al.*, 2009).

The traditional barcoding marker suggested for most animal phyla (Hebert *et al.*, 2003) is a fragment of the mitochondrial gene for subunit I of the cytochrome *c* oxidase (COI). This marker has been used in several phylogenetic studies on North American crayfish (*e.g.*, Taylor and Hardman, 2002; Taylor and Knouft, 2006; Mathews *et al.*, 2008; Dillman *et al.*, 2010), so it is available for a number of potential invaders (although care must be taken when interpreting some data; see Buhay, 2009). The aim of our study is to assemble reference COI sequence data for the North American crayfish known to be present in Europe, to confirm the identification of those species that have not been analysed genetically, and to highlight the potential as well as limitations of DNA barcoding when identifying crayfish invaders.

MATERIAL AND METHODS

We collected publicly available COI sequences from previous studies that analyzed the mitochondrial COI marker in European populations of North American invasive crayfish species (references and GenBank accession numbers are given in Table I). Additionally, we obtained individuals of exotic crayfish populations not yet analyzed genetically: one individual of presumed *Orconectes juvenilis* from the river Dessoubre in France (provided by M. Bramard), two individuals of presumed *O. immunis* from Au am Rhein in Germany and eight individuals from Reiperstwiller in France (collected by C. Chucholl and M. Collas, respectively), and 10 individuals of presumed *Procambarus acutus* from Neder-Hardinxveld in the Netherlands (coll. by D.M. Soes). Individuals from an established marbled crayfish population were captured in the lake Moosweiher in south-western Germany (coll. by C. Chucholl). We also included representative samples of "Old NICS" from Europe in the analysis: *Orconectes limosus*, *Pacifastacus leniusculus* and *Procambarus clarkii*. A summary of all sampled localities is given in Table I.

Reference COI sequences from North American populations of the respective species were mostly obtained from public sources or our unpublished results (Table I). When available, distinct COI haplotypes from North America were chosen to approximately represent the extent of variation of the respective taxon in the native range. Based on the results of Taylor and Knouft (2006), we added sequences of species closely related to "New NICS" to our analysis; only lineages diverging by less than an arbitrarily chosen threshold of 4% were included. When several GenBank sequences of reference species were identical, only one of them was used in the tree. A sequence of *O. rusticus* was also included due to the confusion of European *O. juvenilis* with this species in the past. Additionally, we added a sequence of *O. quinebaugensis* Mathews and Warren, 2008 to complete the *O. virilis* species complex (see Filipová *et al.*, 2010 for details), although its divergence from European *O*. cf. *virilis* slightly exceeded the chosen threshold.

For comparative purposes, we also used sequences of *O. cristavarius* Taylor, 2000 and *O. immunis* from Taylor and Knouft (2006), although these were shown by Buhay (2009) to contain extra insertions close to the 5' end (one or two nucleotides, respectively), and were therefore flagged as "COI-like" sequences in GenBank. Given the position of these problematic regions close to the end of the sequence (and outside of the fragment used in our analysis), and the fact that the amino-acid translation of the preceding sequence does not contain stop codons and agrees with that of other closely related species, we consider these likely to be errors in manual editing and/or a lack of quality control (as suggested by Buhay, 2009) rather that pseudogene sequences. However, careful interpretation of these particular sequences is necessary; therefore these are marked in the tree.

DNA extraction and sequencing of the COI gene fragment followed the methods described in Filipová *et al.* (2010). The DNA was extracted from crayfish leg muscle tissue following a Chelex extraction protocol with proteinase K. The analyzed COI fragment was amplified with the universal primers HCO2198 and LCO1490 (Folmer *et al.*, 1994). Purified PCR products were sequenced in both directions on a capillary sequencer (ABI PRISM 3130), using a BigDye v. 3.1 Terminator Kit. After removal of primer sequences, this resulted in a 658 bp long partial COI sequence. All analyzed sequences were aligned and analyzed in the software MEGA 4.0 (Tamura *et al.*, 2007). The same program was used to construct a neighbour-joining tree and to calculate divergences, using the Kimura 2-parameter model and pairwise deletion of missing data. We provide the neighbour-joining tree (with branch support calculated from 1000 bootstrap pseudoreplications) to show the pattern of COI similarity, as the barcoding approach is based on sequence comparison, not phylogenetic analyses. Furthermore, we assessed the relationship of the taxa included in our analysis by Bayesian inference of phylogeny in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). Independent analyses were performed with settings corresponding to two best-fitting models of evolution, GTR+I+G and HKY+I+G, which were suggested in MrModeltest 2.3 (Nylander, 2004); each analysis consisted of two parallel runs of four Monte Carlo Markov chains run for 3 million generations (20% of which were disregarded as a burn-in phase), with trees sampled every 100 generations. Both models resulted in identical tree topology and almost identical branch support

in Bayesian inference, therefore only results of the latter are provided. Newly obtained sequences were submitted to Genbank (accession numbers JF437985–JF438007).

RESULTS

The patterns of similarity for the COI sequences from European populations of invasive North American crayfish and their American counterparts and relatives are shown in the neighbour-joining tree in Figure 1. The barcoding approach should not be considered a phylogenetic hypothesis; however, the general tree topology agreed well with the results of the Bayesian inference (branch support values are given in Figure 1), and the three genera and main clades within them were recovered by both methods.

As expected, identification of all three "Old NICS", *O. limosus*, *P. leniusculus*, and *P. clarkii*, is completely clear. The maximum intraspecific variation among individuals of European origin included in our study was 0.15% in *O. limosus*, 0.6% in *P. clarkii* and 2.32% in *P. leniusculus*. *Pacifastacus leniusculus* from Europe were relatively variable, all of them were nevertheless more similar to a reference sequence representing the nominate subspecies *P. l. leniusculus* (Dana, 1852) (average divergence 1.13%, maximum 2.48%) than to individuals of the other two recognized subspecies *P. l. trowbridgii* (Stimpson, 1857) and *P. l. klamathensis* (Stimpson, 1857) (average divergences 4.67% and 5.01%, respectively).

The sequenced marbled crayfish individuals from south-western Germany agreed with the morphological identification; they were identical to previously sequenced marbled crayfish, and similar to reference sequences of *P. fallax* from Florida (divergence 0.61% to both US sequences). Similarly, the tested individual from the Dessoubre in eastern France was confirmed to be *O. juvenilis*. It differed by 0.46% from the reference sequence of *O. juvenilis* from Kentucky, USA. The pairwise divergence between the French specimen and *O. rusticus* was 4.42%; divergences to other analysed closely related species, *O. cristavarius*, *O. jeffersoni* (Rhoades, 1944), *O. durelli* (Bouchard and Bouchard, 1995) and *O. sloanii* (Bundy, 1976), ranged from 1.54 to 3.29%.

The match between North American reference sequences and those from the invading populations was substantially lower for *O. immunis*. All analysed European individuals (from both German and French sampling sites) shared the same haplotype, which differed by 2.43 to 3.12% from the two available sequences of *O. immunis* from North America (Ontario and Illinois). Similar divergence values were observed between European *Orconectes* cf. *virilis* and several other known lineages of the *O. virilis* species complex (altogether, these ranged from 1.14% to 4.39%; more details are given in Filipová *et al.*, 2010).

Relatively high variation was observed among sequences from individuals identified as *Procambarus* cf. *acutus* or *P*. cf. *zonangulus*. Sequences of most specimens (9 out of 10) of presumed *Procambarus acutus* from the Netherlands shared the same haplotype, which was very close to the reference sequence of *P. acutus* from Illinois (0.20% divergence). However, the sequence from one individual (GenBank acc. no. JF437987) differed by 2.33% from the remaining Dutch individuals, but was very similar (0.15% divergence) to the sequence of one individual considered to be *P*. cf. *zonangulus* from Louisiana (JF437988). Moreover, we also observed substantial variation among all three individuals supposedly representing *P*. cf. *zonangulus*; with pairwise divergences ranging from 1.38% to 2.97%.

DISCUSSION

DNA barcoding has repeatedly been shown to be highly useful for the identification of invasive species (*e.g.*, Armstrong and Ball, 2005; Saunders, 2009; Van de Wiel *et al.*, 2009; Floyd *et al.*, 2010). The method may also be used to distinguish closely related species or cryptic species complexes (*e.g.*, Bickford *et al.*, 2007; Dillman *et al.*, 2010). This also applies to crayfish, where the COI marker has successfully been used to identify distinct lineages, for example in the

Table I

Summary of sampled localities of studied non-indigenous crayfish species, "Old NICS" (A) and "New NICS" (B), and their close relatives, characteristics of these localities and number of sequenced individuals (n). Sampled areas are Europe (Eur), USA and Canada (Can). Different conspecific haplotypes from the same country or US state are indicated by numerals in brackets. Clades within the Orconectes virilis *complex are labelled according to Mathews* et al. *(2008) and Filipová* et al. *(2010). GenBank accession numbers of new sequences obtained in this study are in bold; the two "COI-like" sequences are indicated by asterisks next to their accession numbers.*

Tableau I

Localités échantillonnées concernant les espèces non-indigènes étudiées, « Old NICS » (A) et « New NICS » (B), et leurs espèces génétiquement proches, ainsi que les caractéristiques de ces localités et le nombre d'individus séquencés (*n*). Les zones échantillonnées sont l'Europe (Eur), les États-Unis (USA) et le Canada (Can). Les différents haplotypes conspécifiques du même pays ou état américain sont indiqués par les chiffres entre parenthèses. Les clades observés au sein de complexe d'*Orconectes virilis* sont indiqués conformément à Mathews *et al.* (2008) et Filipová *et al.* (2010). Les numéros d'accès de GenBank des nouvelles séquences obtenues pour cette étude sont notés en caractère gras et les deux séquences « COI-like » par les astérisques à côté de leur numéro d'accès de GenBank.

	Species	Area	Country/ US state	Locality	Region/ US county	Type of waterbody	Sampling date	Latitude	Longitude	*n*	GenBank acc. no.
A	***Orconectes limosus***										
	O. limosus	Eur	Czech Republic (1)	Malše, České Budějovice	South Bohemia	river	12 Sep. 2005	48° 58' N	14° 29' E	1	**JF437991**
	O. limosus	Eur	Czech Republic (2)	Prudník, Osoblaha	Silesia	river	27 Oct. 2006	50° 18' N	17° 43' E	1	**JF437993**
	O. limosus	Eur	France	Vouneuil-sous-Biard	Poitou-Charentes	brook	13 Jul. 2006	46° 34' N	0° 16' E	1	**JF437992**
	O. limosus	USA	Maine	Pleasant River	Oxford Co.	river	Nov. 2009	43° 46' N	70° 26' W	1	**JF437994**
	O. limosus	USA	New York	East Branch Delaware River	Delaware Co.	river	1 Oct. 2002	41° 58' N	75° 11' W	1	AY701199
	O. obscurus	USA	New York	Nine Mile Creek	Oneida Co.	brook	N/A	N/A	N/A	1	AF474354
	Procambarus clarkii										
	P. clarkii	Eur	France (1)	Rochechevreux, Lignac	Centre	pond	summer 2010	46° 28' N	1° 13' E	1	**JF438004**
	P. clarkii	Eur	France (2)	Rochechevreux, Lignac	Centre	pond	summer 2010	46° 28' N	1° 13' E	1	**JF438003**
	P. clarkii	Eur	France (3)	Givrezac, Le Tâtre	Poitou-Charentes	pond	2005	45° 24' N	0° 12' E	1	**JF438002**
	P. clarkii	USA	Illinois	Horseshoe Lake	Alexander Co.	lake	N/A	N/A	N/A	1	AY701195
	P. clarkii	USA	Louisiana	New Orleans	New Orleans parish	pond	2005	29° 57' N	90° 5' W	1	**JF438001**
	Pacifastacus leniusculus										
	P. l. leniusculus	Eur	Czech Republic	Jedlová, Litomyšl	East Bohemia	pond	Apr., Oct. 2006	49° 39' N	16° 18' E	1	**JF437995**
	P. l. leniusculus	Eur	Hungary	Gyöngyös, Köszeg	Vas	river	summer 2005	47° 23' N	16° 32' E	1	**JF437996**
	P. l. leniusculus	Eur	Great Britain	Teil Burn, Five	Scotland	brook	Jun. 2009	56° 13' N	3° 09' W	1	**JF437997**
	P. l. leniusculus	USA	Oregon	Upper Williamson River	Klamath Co.	river	May 2003	42° 51' N	121° 28' W	1	**JF437998**
	P. l. klamathensis	USA	California	Lower Klamath River	Humboldt Co.	river	May 2004	41° 35' N	123° 32' W	1	**JF437999**
	P. l. trowbridgii	USA	Oregon	Siuslaw River	Lane Co.	river	2003	44° 04' N	123° 53' W	1	**JF438000**

Table I
Continued.

Tableau I
Suite.

Species	Area	Country/ US state	Locality	Region/ US county	Type of waterbody	Sampling date	Latitude	Longitude	*n*	GenBank acc. no.
B Orconectes immunis										
O. immunis	Eur	Germany	Rhein, Au am Rhein	Baden-Württemberg	river	14 Oct. 2010	48° 58' N	8° 12' E	2	**JF438005**
O. immunis	Eur	France	Rothbach, Reiperstwiller	Bas-Rhin	brook	8 Nov. 2010	48° 56' N	7° 30' E	8	**JF438006**
O. immunis	USA	Illinois	Goose Creek	Piatt Co.	brook	N/A	N/A	N/A	1	AY701220
O. immunis	Can	Ontario	N/A	N/A	N/A	N/A	N/A	N/A	1	DQ882095*
Orconectes juvenilis and related species										
O. juvenilis	Eur	France	Dessoubre River, Rosureux	Franche-Comté	river	2008	47° 13' N	6° 41' E	1	**JF437985**
O. juvenilis	USA	Kentucky	Rockcastle River	Rockcastle Co.	river	N/A	N/A	N/A	1	AF474352
O. sloanii	USA	Indiana	Six Mile Creek	Jennings Co.	brook	N/A	N/A	N/A	1	AY701197
O. jeffersoni	USA	Kentucky	Mid. Fk. Beargrass Creek	Jefferson Co.	brook	N/A	N/A	N/A	1	AF474351
O. cristavarius	USA	Kentucky	Millers Creek	Lee Co.	brook	N/A	N/A	N/A	1	AY701230*
O. durelli	USA	Kentucky	Trammel Fk.	Allen Co.	brook	N/A	N/A	N/A	1	AF474348
Orconectes rusticus										
O. rusticus	USA	Kentucky	Ohio River	Trimble Co.	river	N/A	N/A	N/A	1	AY701249
Orconectes virilis complex										
O. cf. *virilis*	Eur	Great Britain	River Lee system	Greater London	river	8 Nov. 2009	51° 36' N	0° 02' W	3	FJ608576
O. cf. *virilis*	Eur	Netherlands	Kanis, Woerden	Utrecht	ditch	20 Aug. 2008	52° 08' N	4° 53' E	1	FJ608578
O. virilis s.s. (clade 1)	USA	Massachusetts	Ware River, Rutland	Worcester Co.	river	2005–2007	42° 26' N	71° 58' W	1	EU442752
O. cf. *virilis* (clade 3)	USA	Kansas	King's Creek, Manhattan	Riley Co.	brook	2005–2007	39° 07' N	96° 36' W	1	EU442727
O. cf. *virilis* (clade 4)	USA	Kansas	Mission Creek, Dover	Shawnee Co.	brook	2005–2007	38° 58' N	95° 56' W	1	EU442740
O. cf. *virilis*	USA	Iowa	Squaw Creek, Cedar Rapids	Linn Co.	brook	29 May 2006	41° 58' N	91° 40' W	1	FJ608575
O. quinebaugensis	USA	Massachusetts	Puffer's Pond, Amherst	Hampshire Co.	pond	2005–2007	42° 25' N	72° 31' W	1	EU442772
O. deanae	USA	New Mexico	Conchas Lake	San Miguel Co.	lake	N/A	N/A	N/A	1	EU442723
O. nais	USA	Oklahoma	Canadian River	Cleveland Co.	river	N/A	N/A	N/A	1	EU442721

Table I
Continued.

Tableau I
Suite.

Species	Area	Country/ US state	Locality	Region/ US county	Type of waterbody	Sampling date	Latitude	Longitude	*n*	GenBank acc. no.
***Procambarus acutus/zonangulus* complex**										
P. cf. *acutus*	Eur	Netherlands	Neder-Hardinxveld	Zuid-Holland	ditch	13 Jan. 2010	51° 50' N	4° 52' E	9	**JF437986**
P. cf. *zonangulus*	Eur	Netherlands	Neder-Hardinxveld	Zuid-Holland	ditch	13 Jan. 2010	51° 50' N	4° 52' E	1	**JF437987**
P. cf. *acutus*	USA	Illinois	trib. Cypress Creek	Union Co.	brook	N/A	N/A	N/A	1	AF474366
P. cf. *zonangulus*	USA	Louisiana (1)	research pond	Baton Rouge Parish	pond	Apr. 2010	30° 22' N	91° 11' W	1	**JF437988**
P. cf. *zonangulus*	USA	Louisiana (2)	research pond	Baton Rouge Parish	pond	Apr. 2010	30° 22' N	91° 11' W	1	**JF437989**
P. cf. *zonangulus*	USA	Louisiana (3)	research pond	Baton Rouge Parish	pond	Apr. 2010	30° 22' N	91° 11' W	1	**JF437990**
Procambarus fallax										
Marmorkrebs	Eur	Germany (1)	Lake Moosweiher, Freiburg im Breisgau	Baden-Württemberg	lake	14 Oct. 2010	48° 02' N	7° 48' E	2	**JF438007**
Marmorkrebs	Eur	Germany (2)	Elligastbach, Zabeltitz-Uebigau	Saxony	brook	11 Oct. 2009	51° 20' N	13° 34' E	1	HM358011
P. fallax	USA	Florida (1)	Everglades	N/A	N/A	N/A	N/A	N/A	1	HQ171456
P. fallax	USA	Florida (2)	Everglades	N/A	N/A	N/A	N/A	N/A	1	HQ171455

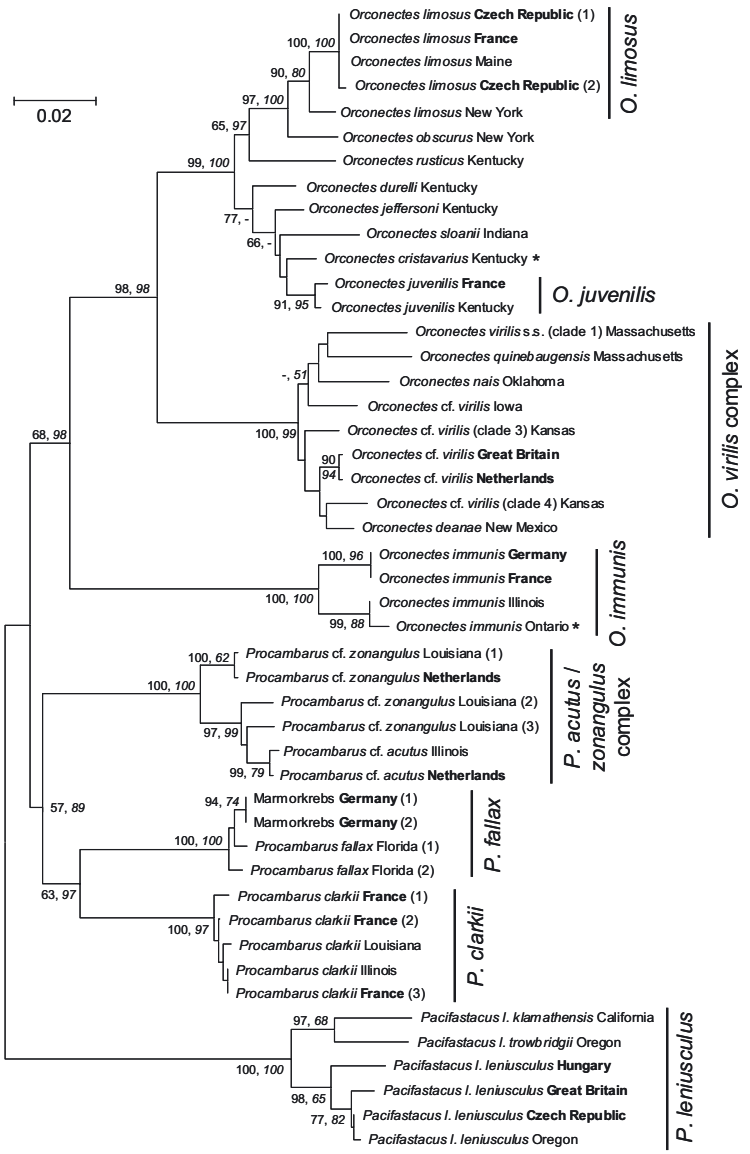

Figure 1

Neighbour-joining tree showing variation in the cytochome c *oxidase subunit I of exotic North American crayfish collected in Europe (countries highlighted in bold), together with reference sequences of these and other closely related species from North America. Numbers at selected nodes indicate the branch support in the neighbour joining (first value) and Bayesian inference (second value, in italics) analyses, respectively; only values above 50% are shown. Scale indicates 2% divergence. Different conspecific haplotypes from the same country or US state are indicated by numerals in brackets; the two "COI-like" sequences are marked by asterisks. Clades within the* O. virilis *complex are labelled according to Mathews* et al. *(2008) and Filipová* et al*. (2010).*

Figure 1

Arbre *neighbour joining* montrant une variation de cytochrome *c* oxydase sous-unité I (COI) chez les écrevisses d'Amérique du Nord échantillonnées en Europe (pays notés en caractère gras), ainsi que les séquences de référence de ces espèces et des espèces proches provenant d'Amérique du Nord. Les valeurs des nœuds sélectionnés indiquent le support des branches générées par les analyses de *neighbour joining* (première valeur) et l'inférence bayésienne (seconde valeur, en italique) ; seules les valeurs au-dessus de 50 % sont visualisées. L'échelle représente 2 % de divergence. Les différents haplotypes conspécifiques du même pays ou d'un état américain sont indiqués par les chiffres entre parenthèses et les deux séquences « COI-like » par les astérisques à côté de leur numéro d'accès de GenBank. Les clades au sein de complexe d'*O. virilis* sont marqués conformément à Mathews *et al.* (2008) et Filipová *et al.* (2010).

O. virilis complex (Mathews *et al.*, 2008). An important advantage of the method is the possibility to also identify individuals or development stages lacking characteristics needed for morphological discrimination (Briski *et al.*, 2010; Floyd *et al.*, 2010). North American cambarid crayfish, particularly males, are usually seasonally dimorphic (Hobbs, 1989), and their morphological determination is often based on gonopods of males of reproductively active form I (Hobbs, 1972; Holdich and Reeve, 1988; Hobbs, 1989; Taylor and Knouft, 2006). If individuals of this form are not available, molecular markers may be applied to identify samples.

DNA barcoding may also facilitate the identification of invasive crayfish for researchers (or other stakeholders) that lack experience in morphological analyses in this group. This might be particularly useful in Western and Central Europe where all "New NICS" have been recorded so far (Holdich *et al.*, 2009): the number of native crayfish species in that region is very low, and their identification is relatively easy. On the contrary, the source region of most new invaders, North America, has very high crayfish species richness (Hobbs, 1989). Many American crayfish are genetically as well as morphologically similar (*e.g.*, Taylor and Knouft, 2006; Mathews *et al.*, 2008), so reliable identification requires substantial expertise usually not available in invaded ranges overseas. Of course, molecular-based identification of crayfish invaders is not limited to Europe (for a recent example from Madagascar, see Jones *et al.*, 2009), and may also be useful within North America where crayfish invasions are common as well (Hobbs *et al.*, 1989).

We did not expect any problems with COI-based identification of the "Old NICS", *O. limosus*, *P. clarkii* and *P. leniusculus*. However, different levels of intraspecific mtDNA variation found in European populations of these species could be expected due to different numbers of introduced individuals of these invaders brought to Europe and the number of introduction events (Souty-Grosset *et al.*, 2006; Barbaresi *et al.*, 2007; Filipová *et al.*, 2009). Analysis of the "New NICS" confirmed the identification of samples from the established marbled crayfish population from Germany (Chucholl and Pfeiffer, 2010) and the *O. juvenilis* population from France (Chucholl and Daudey, 2008). However, analyses of two other taxa included in our study, *O. immunis* from Germany and France, and presumed *Procambarus acutus* from the Netherlands, resulted in ambiguous patterns, similarly as was the case for previously studied European populations considered to be *O. virilis* (Filipová *et al.*, 2010).

The high divergence between the samples of *O. immunis* from Europe and North America corresponds to divergences found among distinct orconectid species (Figure 1; see also Mathews *et al.*, 2008; Dillman *et al.*, 2010), although comparable or even higher intraspecific variation is observed in other crayfish genera, such as *Austropotamobius* in Europe (Trontelj *et al.*, 2005) and apparently also *Pacifastacus* in North America (this study). Variation in the North American range of *O. immunis* should be therefore studied to test whether this taxon is a cryptic species complex, as has recently been shown for *O. virilis* (Mathews *et al.*, 2008), or whether such levels of divergence are within the range of intraspecific variation.

Similarly, patterns of variation observed among American sequences representing *P*. cf. *acutus* and *P*. cf. *zonangulus* confirm that the taxonomy of this species complex needs further analyses. Difficulties in the conclusive identification of white river crayfish found in the Netherlands have already been mentioned by Holdich *et al.* (2009). Our analysis shows that the local population is not genetically homogeneous and might actually be a mixed population of two species of the white river crayfish complex: although sequences from most Dutch specimens were nearly identical to *P*. cf. *acutus* sequence from Illinois, one of them was divergent, very similar to *P*. cf. *zonangulus* from Louisiana. High variation within white river crayfish from the Midwestern USA has been demonstrated by Fetzner *et al.* (1997), based on allozyme markers. Future analysis of the white river crayfish complex should focus on morphological and genetic variation in their native range (including molecular characterization of both species from the regions of their type localities). However, further research on the invasive population in the Netherlands containing a mixture of haplotypes may also provide important insights; for example, the level of reproductive isolation between divergent lineages may be studied in such localities by nuclear markers.

These examples also show the importance of comparing newly analysed COI sequence data to appropriate reference sequences. Missing data on relevant taxa may prevent identification, while the presence of a closely related congener but absence of a conspecific in the reference data set may lead to misidentification of the invader (Armstrong and Ball, 2005). Before reaching conclusions on the taxonomic status of seemingly divergent lineages, more data on genetic variation within the concerned taxon should be obtained from the original distribution range. Analyses of nuclear markers may also be desirable to avoid erroneous interpretation of results. Even clearly divergent haplogroups detected by mtDNA barcoding may not necessarily represent biological species (*e.g.*, Dasmahapatra *et al.*, 2009); on the other hand, barcoding may also underestimate the real number of species (*e.g.*, Whitworth *et al.*, 2007). In any case, discoveries of unusual genotypes in invaded ranges may stimulate further research into those particular invasive taxa. We hope this may also be the case for the "New NICS" in Europe, especially as white river crayfish are important for aquaculture in the USA (Huner *et al.*, 1994; Romaire *et al.*, 2005).

In general, DNA barcoding seems to be a useful tool not only for the rapid identification of exotic crayfish but also for a better understanding of their diversity, especially if combined with detailed morphological examinations. Genetic data may be also useful for testing whether newly recorded populations are likely to have been introduced from already established ones, or whether they result from new introductions; such information is important for determining the most important pathways of invasions (Floyd *et al.*, 2010). As most "New NICS" found in Europe are supposed to come from the aquarium trade, knowledge on the genetic variation of specimens available in aquarium cultures could provide useful information on their origin. DNA barcoding can also be applied to well-preserved voucher specimens of uncertain taxonomic status, which might reveal the long-term yet unrecorded presence of invasive species in the area (Humble *et al.*, 2009).

It is likely that more crayfish species will be introduced to Europe in the future, in particular through the aquarium trade or aquaculture (Holdich *et al.*, 2009). As the misidentification of invaders may have negative consequences for risk assessment and future management strategies (Bickford *et al.*, 2007; Floyd *et al.*, 2010), fast and reliable identification of newly recorded invasive crayfish populations is important. The results of our study therefore contribute not only to knowledge on the present diversity of alien crayfish in Europe, but also provide reference data needed for future barcoding-based identification of NICS.

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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.

Prevalence of the crayfish plague *Aphanomyces astaci* in populations of signal crayfish *Pacifastacus leniusculus* in France

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Abstract

The pathogen of the crayfish plague *Aphanomyces astaci* first appeared in Europe in 1859 and nowadays it still causes mass mortalities of native European crayfish. Its spread across the continent is facilitated especially by invasive North American crayfish species which serve as its reservoir. In France, multiple cases of native crayfish mortalities were suggested to be connected with the presence of the signal crayfish *Pacifastacus leniusculus*, which is highly abundant in the country. It shares similar habitat as the native white-clawed crayfish, *Austropotamobius pallipes* and, if infected, the signal crayfish might therefore easily transmit the pathogen to the native species. The aim of our study was to investigate the prevalence of *A. astaci* in French signal crayfish populations and thus to evaluate the danger they represent for local populations of native crayfish. Using the quantitative TaqMan minor groove binder (MGB) real-time PCR, we have analysed 513 individuals of *Pacifastacus leniusculus* from 45 French populations. Altogether, 20% of these crayfish were found to be infected, and the pathogen was detected in more than a half of studied populations. Local prevalence varied significantly, ranging from 0% up to 80%. Our results confirm that the widespread signal crayfish serves as a reservoir of *Aphanomyces astaci* in France and represents therefore a serious danger for native crayfish species, especially the white-clawed crayfish. Additional analysis of several individuals of other non-indigenous crayfish species (*Orconectes limosus*, *O. immunis* and *Procambarus clarkii*) revealed infections among two of these, *O. immunis* and *P. clarkii*. Prevalence in other introduced crayfish should therefore be investigated as they may also contribute to the transmission of the pathogen in the country.

Key-words: crayfish plague, *Aphanomyces astaci*, signal crayfish, France

Introduction

For more than 150 years, native crayfish in Europe are being decimated by the crayfish plague, a disease caused by an oomycete *Aphanomyces astaci* (Schikora, 1903). The first European outbreak of the plague was recorded in 1859 in northern Italy (Holdich 2003), the second focus of the disease appeared in France in 1874 at Plateau des Langres (Alderman 1996). In the following decades, the pathogen continued to spread to other European countries (Alderman 1996, Holdich 2002, Souty-Grosset et al. 2006). At present, the known carriers of the pathogen in Europe are non-indigenous North American crayfish species, especially three most widespread ones: the spiny-cheek crayfish *Orconectes limosus*, the signal crayfish *Pacifastacus leniusculus* and the red swamp crayfish *Procambarus clarkii* (Henttonen and Huner 1999). Their presence in European waters facilitates the persistence and spread of the parasite and further contributes to mortalities of native crayfish (e.g. Kiszely 2004, Kozubíková et al. 2008, Oidtmann et al. 1999, Souty-Grosset et al. 2006, Vennerström et al. 1998). In France, the impact of these mortalities was enormous: before the disease stroke in French waters, more than 15 thousands of crayfish were sent to Paris each day (Raveret-Wattel 1885). Later, the amount of crayfish sold for consumption in Paris remained high but most of these crayfish had to be imported from abroad (Raveret-Wattel 1885).

Largest mass mortalities of native crayfish in France took place between the 1870s and 1912 (Machino and Diéguez-Uribeondo 1998, Raveret-Wattel 1885), but after a relatively calm period, new outbreaks of crayfish plague have been reported in the country since the 1990s (Collas and Salek 2002, Edgerton et al. 2004, Machino and Diéguez-Uribeondo 1998). Some of these mortalities of native species were suspected to be connected with the presence of invasive signal crayfish *Pacifastacus leniusculus* (Collas and Salek 2002, Neveu 1998a, 1998b, 2000, 2002). The link between signal crayfish presence and crayfish plague spread is apparent also in other European countries (e.g. Bohman et al. 2006, Diéguez-Uribeondo 2006, Machino and Diéguez-Uribeondo 1998, Vennerström et al. 1998).

The signal crayfish was introduced to France in 1972 from Sweden, and in 1974 more individuals were brought directly from North America (Lake Tahoe and Lake Donner in California); this was followed by numerous secondary introductions (Arrignon et al. 1999). The species is now widely distributed in France (Arrignon et al. 1999, Collas et al. 2007, Holdich 2002, Machino 1999, Souty-Grosset et al. 2006, Vigneux 1997). In 2006, it was found at about 1000 sites in 73 out of 96 French metropolitan departments (Collas et al. 2007). Although several other non-indigenous crayfish species are found in France, *Pacifastacus leniusculus* represents the largest threat to native species, especially the white-

clawed crayfish *Austropotamobius pallipes* (Collas et al. 2007). Signal crayfish inhabit similar habitats in headwaters of rivers and may therefore easily get into contact with *A. pallipes* populations (Bramard et al. 2006), which facilitates the transmission of the pathogen to the native species if the invasive one is infected.

The white-clawed crayfish is the most abundant native crayfish in France. In 2006, it was still found at 2249 sites in 76 out of 96 metropolitan departments (Collas et al. 2007). However, the number of its populations has significantly decreased recently, and the crayfish plague represents one of the major factors which contribute to the decline of *A. pallipes* in France (Bramard et al. 2006). Moreover, Collas et al. (2007) showed that 89% of crayfish mortalities recorded in France between years 2001 and 2006 affected *Austropotamobius pallipes*, while only the remaining 11% of mortalities concerned other species.

Despite the substantial impact the crayfish plague has on native crayfish species, reliable information on the presence of its pathogen *A. astaci* in European waters is rather scattered. For many years, mortalities of native crayfish, if noticed, suggested the presence of the disease. However, the identity of the pathogen was often assumed rather than confirmed, due to difficulties with its cultivation and ambiguous morphological characteristics (Alderman and Polglase 1986, Oidtmann et al. 2002). Recently, several methods of molecular detection of *Aphanomyces astaci* have been developed which do not require cultivation (Hochwimmer et al. 2009, Oidtmann et al. 2004, 2006, Vrålstad et al. 2009). Very low quantities of the pathogen DNA in the sample are detectable by these methods, enabling large-scale screening of populations of invasive crayfish from different European countries. So far, the largest datasets have been obtained by Kozubíková et al. (2009, 2011) who studied crayfish plague prevalence in more than 300 individuals of *Orconectes limosus* and more than 100 individuals of *Pacifastacus leniusculus* from Central Europe. In other studies, only one or a few populations were analysed (e.g. Pârvulescu et al. 2012, Skov et al. 2011, Vrålstad et al. 2011).

So far, no data on the prevalence of the crayfish plague in non-indigenous crayfish populations in France have been available. In the present study, we therefore tested numerous French populations of *Pacifastacus leniusculus* and also a few individuals of other alien crayfish species for the presence of *Aphanomyces astaci*. A quantitative TaqMan minor groove binder (MGB) real-time polymerase chain reaction (PCR) designed by Vrålstad et al. (2009) was chosen for our analyses, as it proved to be the most sensitive of available detection methods, and highly specific to *A. astaci* (Kozubíková et al. 2011, Tuffs and Oidtmann 2011). Our aim was to evaluate the threat signal crayfish populations in France represent to native crayfish species. Data on the plague prevalence could then allow targeting invasive crayfish populations with highest infection ratio for potential future

eradication trials, and native crayfish populations which are at highest risk that could be translocated to safer areas, i.e., the “ark sites” (Peay 2009). Thus, data on the distribution and prevalence of crayfish plague may contribute to better efficiency of the conservation management of native species, in particular *Austropotamobius pallipes*, in the country.

Material and methods

In total, 513 individuals of signal crayfish *Pacifastacus leniusculus* were sampled from 45 localities in France (Table 1, Fig. 1) by hand or by electrofishing. Most individuals came from running waters, especially brooks and several rivers. In addition, several individuals of other non-indigenous species were analysed. These were 19 individuals of *Orconectes limosus* from two populations, seven individuals of *O. immunis* from one population, and two individuals of *Procambarus clarkii* from one population (Table 1). Sampled populations, their characteristics and the number of analysed individuals from each population are summarised in Table 1.

Captured crayfish were stored in 96% ethanol. Tissue from one half of the soft abdominal cuticle and one uropod was dissected from each crayfish using sterile tools. Dissected tissues from one individual were collected in a single 1.5 ml tube, dried and stored in a deepfreezer at -80°C. Before further processing, 360 µl of the Buffer ATL from the DNeasy tissue kit (Quiagen) was added to the unfrozen dissected material. The mixture was then crushed by one scoop (ca 50 µl) of stainless steel beads (1.6 mm diameter) using BBX24B Bullet Blender (Next Advance) for 10 min at maximum speed. DNA extractions from the crushed cuticle then followed the rest of the spin-column protocol of the DNeasy tissue kit, in double volume (i.e., with 40 µl of the proteinase K solution and 400 µl of the Buffer AL).

Isolated material was then tested for the presence of *Aphanomyces astaci* by the quantitative TaqMan MGB real-time PCR designed by Vrålstad et al. (2009), using the LightCycler® 480 Instrument (Roche). A 59 bp fragment of the internal transcribed spacer (ITS) region of *A. astaci* nuclear rDNA was amplified using primers AphAstITS-39F (5’-AAG GCT TGT GCT GGG ATG TT-3‘) and AphAstITS-97R (5’-CTT CTT GCG AAA CCT TCT GCT A-3’), and quantified with the pathogen-specific TaqMan® minor groove binder (MGB) probe AphAstITS-60P (5‘-6-FAM-TTC GGG ACG ACC C-MGBNFQ-3‘). The total 25 µl reaction volume consisted of a 2x Universal PCR Master Mix (Applied Biosystems), both primers (500 nM each), TaqMan MGB-Probe (200 nM), nuclease free water, and template DNA (around 20 ng/µl). The PCR program consisted of one cycle of 10 min at 95°C, 50 cycles of 15 sec at 95°C and 60 sec at 58°C and one final cycle of 60 sec at 40°C. In each run, two replicates of four different standards that served as positive controls and ensured

the comparability of different runs. The quantity of the pathogen DNA in these standards, expressed in PCR forming units (PFU), were 3×4^{10}, 3×4^{8}, 3×4^{4} and 3×4^{2}, respectively (Vrålstad et al. 2009). Two negative controls (which remained negative in all runs) were included in each run to detect possible contamination. For each isolate, undiluted and 10-fold diluted replicate was analysed to test for the impact of inhibition which might influence the efficiency of detection (Strand et al. 2011, Vrålstad et al. 2009). When some effects of inhibition were occasionally detected (mostly in samples with low agent level), the PFU values were estimated as described in Kozubíková et al. (2011).

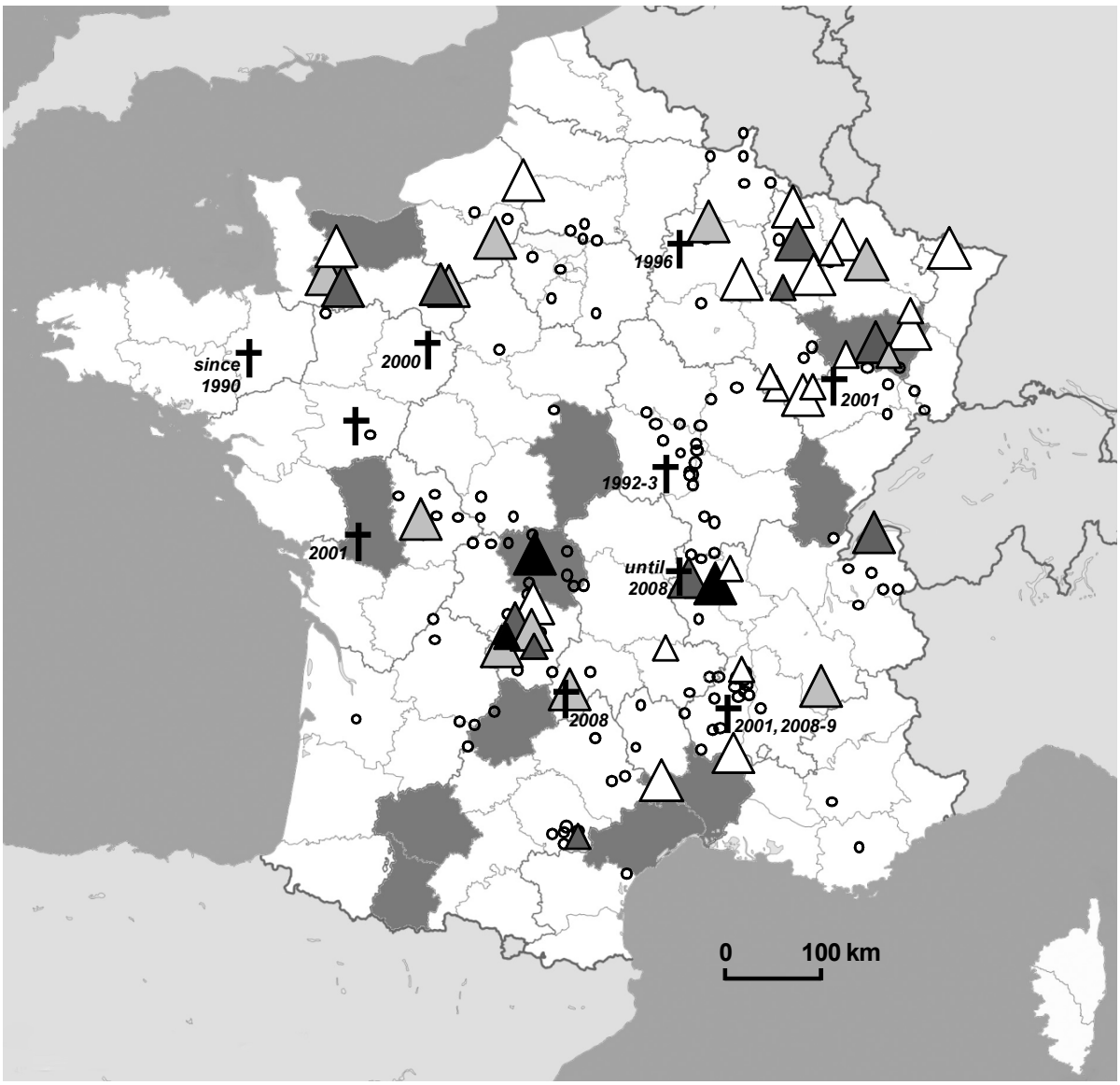
Based on their PFU values, samples were classified into semiquantitative categories of pathogen load, ranging from A0 (no traces of *A. astaci* DNA) to A7 (extremely high amount of *A. astaci* DNA in the sample), as proposed by Vrålstad et al. (2009). Only individuals with agent level A2 and higher were considered infected. Agent level A1 falls below the limit of detection of the method (corresponding to 5 PFU) and may not only indicate trace amounts of pathogen DNA, but also false positives or minor contamination during analyses. Therefore, agent level A1 should not be considered a confirmation of the presence of *A. astaci* in the sample (Kozubíková et al. 2011, Vrålstad et al. 2009).

Results

The number of infected individuals in sampled populations, agent levels detected in infected specimens and the crayfish plague prevalence for each population are summarised in Table 1. Some of the sampled signal crayfish came from localities where the presence of the species has not been reported so far, and thus enlarge the known range of its distribution in France (Fig. 1).

In total, 103 signal crayfish (20%) from 24 populations (53%) were found to be infected (with agent level A2 or higher) (Table 1). The pathogen prevalence in studied populations was variable, ranging from 0% to 80% (Table 1). In 322 signal crayfish, no traces of *Aphanomyces astaci* were found (agent level A0), and 88 individuals were assigned to agent level A1 (i.e., weak signal not considered as positive pathogen detection). Most samples (73 individuals) that tested positive contained low amount of pathogen DNA (agent level A2), the agent level A3 was found in 28 individuals and A4 in two individuals (Table 1).

Fig 1. Map showing approximate locations of sampled localities of *Pacifastacus leniusculus* in France (triangles). Prevalence of crayfish plague in sampled populations is expressed by the colour of triangles: no traces of pathogen (0%, white triangles), slightly infected (1-30%, light grey triangles), medium infected (31-60%, dark grey triangles) and heavily infected population (61-100%, black triangles). The size of triangles indicates the number of analysed individuals in the respective population (small triangle – less than nine individuals, large triangle – ten or more individuals). Empty circles show the distribution of *P. leniusculus* in France (according to Arrignon et al. 1999, Souty-Grosset et al. 2006, Vigneux 1997 and L. Filipová, unpublished data). Black crosses with years indicate mortalities of native crayfish most likely caused by *A. astaci*, reported in France since the 1990s (Collas and Salek 2002, Machino and Diéguez-Uribeondo 1998, Neveu 1998a, 1998b, 2000, Neveu and Bachelier 2003, Papin 2000, T. Duperray, and T. Pantarotto, pers. comm.). Crayfish-plague associated mortalities were reported between 2001 and 2005 from departments highlighted in grey (according to Collas et al. 2007).



Distribution of *P. leniusculus* populations with highest plague prevalence (over 50%) was rather scattered, as these were located in Limousin, Rhône-Alpes, Basse-Normandie and Meuse regions (Table 1, Fig. 1). In some regions, infected individuals were found in the majority of local populations, such as in Limousin (6 infected out of 7 analysed populations), Rhône-Alpes (4/6), and Basse-Normandie (4/5). On the other hand, very low numbers of individuals infected by the plague were found in populations from the Lorraine region, where only 7 individuals from 121 analysed signal crayfish were infected (in 3 out of 11 analysed populations), or in populations from Champagne-Ardenne with five infected out of 68 tested individuals, coming from a single population in La Vesle (Prunay, Marne) out of 6 analysed (Table 1). In Languedoc-Rousillon, none of the signal crayfish tested positive for the presence of *A. astaci*, however, only 20 individuals from two populations were analysed in this region.

The real-time PCR analyses confirmed the presence of *A. astaci* also in two out of three other crayfish species analysed. None of the analysed individuals of *Orconectes limosus* from any of the sampled populations (Indre and Rhône-Alpes regions) showed traces of the pathogen. However, two out of seven tested individuals (29%) of *Orconectes immunis* from Alsace were infected, as well as one out of two analysed individuals of *Procambarus clarkii* from Indre (Table 1).

Table 1 Summary characteristics, and results of *Aphanomyces astaci* detection in 45 analysed French populations of signal crayfish *Pacifastacus leniusculus* and four additional populations of other non-indigenous crayfish species (*Procambarus clarkii*, *Orconectes limosus* and *O. immunis*). Number of analysed and infected individuals per population, and the prevalence of the crayfish plague pathogen *Aphanomyces astaci* are given for each population. Numbers in brackets in the last column indicate the number of infected specimens with that particular agent level (individuals with agent levels A0 and A1 are not given). Where no number is provided, the same agent level was detected in all infected individuals from that population.

Species	Locality	Closest settlement	Region	Department	Latitude	Longitude	Type of waterbody	Sampling date	Individuals analysed	Individuals infected	Prevalence	Agent level in infected inds.
P. leniusculus												
	La Cère	Sansac de Marmiesse	Auvergne	Cantal	44° 53' N	2° 22' E	river	6 Sep 2010	10	2	**20%**	A2
	La Senouire	St. Pal de Senouire	Auvergne	Haute Loire	45° 15' N	3° 39' E	brook	26 Jul 2010	5	0	0%	-
	Ruisseau du Parc	Mesnil-Auzouf	Basse-Normandie	Calvados	48° 59' N	0° 43' W	brook	31 Mar 2010	18	0	0%	-
	Varenne	Saint-Bômer-les-Forges	Basse-Normandie	Orne	48° 37' N	0° 36' W	brook	2 Sep 2009	14	8	**57%**	A2(6), A3(2)
	Egrenne	Beauchêne	Basse-Normandie	Orne	48° 41' N	0° 45' W	brook	2 Sep 2009	15	4	**27%**	A2
	Sarthon	St Denis-sur-Sarthon	Basse-Normandie	Orne	48° 27 N	0° 03' W	brook	Oct 2009	14	4	**29%**	A2
	Sarthon	Rouperroux	Basse-Normandie	Orne	48° 32 N	0° 05' W	brook	Oct 2009	16	7	**44%**	A2(6), A3(1)
	Ruisseau de la Côte Saint-Gilles	Saint Aubin sur Gaillon	Haute-Normandie	Eure	49° 08' N	1° 21' E	brook	2 nov 2009	12	1	**8%**	A2
	Mesangueville	Dampierre-en-Bray	Haute-Normandie	Seine-Maritime	49° 32' N	1° 40' E	brook	17 Sep 2009	10	0	0%	-
	Le Vannon	Genévrieres	Champagne-Ardenne	Haute-Marne	47° 42' N	5° 36' E	brook	16-17 Sep 2009	14	0	0%	-
	Petits Crots	Poinson-lès-Fayl	Champagne-Ardenne	Haute-Marne	47° 45' N	5° 35' E	brook	24 Sep 2009	5	0	0%	-
	Ource	Colmier-le-Bas	Champagne-Ardenne	Haute-Marne	47° 46' N	4° 57' E	brook	18 Sep 2009	5	0	0%	-
	Aube	Rouvres sur Aube	Champagne-Ardenne	Haute-Marne	47° 51' N	5° 00' E	brook	2 Sep 2009	5	0	0%	-
	Bruxenelle	Plichancourt	Champagne-Ardenne	Marne	48° 45' N	4° 40' E	brook	3 Sep 2009	22	0	0%	-
	La Vesle	Prunay	Champagne-Ardenne	Marne	49° 11' N	4° 11' E	river	2 Sep 2009	17	5	**29%**	A2
	la Foux, Pont de la Mouline	Lanuejols	Languedoc-Rousillon	Gard	44° 07' N	3° 25' E	brook	28 May 2009	10	0	0%	-
	Moze, Moulin du Deloly	St. Julien-de-Peyrolas	Languedoc-Rousillon	Gard	44° 17' N	4° 34' E	brook	11 Nov 2009	10	0	0%	-
	La Mayne	Concèze	Limousin	Corrèze	45° 21' N	1° 21' E	brook	2 Aug 2010	12	1	**8%**	A2
	La Vézère	Uzerche	Limousin	Corrèze	45° 25' N	1° 34' E	brook	3 Aug 2010	5	4	**80%**	A2(3), A3(1)
	La Douyge	St. Augustin	Limousin	Corrèze	45° 25' N	1° 51' E	brook	29 Jul 2010	9	4	**44%**	A2
	La Maulde	St Martin Chateau	Limousin	Creuse	45° 51' N	1° 49' E	brook	6 Jul 2010	12	0	0%	-
	Cherpont	Sainte-Feyre	Limousin	Creuse	46° 09' N	1° 57' E	brook	4 Jun 2009	15	11	**73%**	A2(7), A3(4)

Table 1 (continued)

Species	Locality	Closest settlement	Region	Department	Latitude	Longitude	Type of waterbody	Sampling date	Individuals analysed	Individuals infected	Prevalence	Agent level in infected inds.
P. leniusculus												
	La Petite Briance	St Germain les Belles	Limousin	Haute Vienne	45° 37' N	1° 30' E	brook	3 Jun 2010	9	3	**33%**	A2(2), A3(1)
	La Grande Briance	Croiselle sur Briance	Limousin	Haute Vienne	45° 36' N	1° 36' E	brook	29 Jul 2010	14	2	**14%**	A2
	Longeau	Allamont	Lorraine	Meurthe-et-Moselle	49° 07' N	5° 48' E	brook	25 Sep 2009	5	0	0%	-
	Orne	Hatrize	Lorraine	Meurthe-et-Moselle	49° 12' N	5° 55' E	river	26 Aug 2009	15	0	0%	-
	Othain	Petit-Failly	Lorraine	Meurthe-et-Moselle	49° 26' N	5° 29' E	brook	27 Aug 2009	18	0	0%	-
	pond Claveau on Herbas brook	Cirey-sur-Vezouze	Lorraine	Meurthe-et-Moselle	48° 36' N	6° 58' E	pond	4 Oct 2010	8	0	0%	-
	Seigneulle	St. Maurice	Lorraine	Meuse	49° 01' N	5° 41' E	brook	1 Oct 2009	15	0	0%	-
	Zinzel du Nord	Baerenthal	Lorraine	Moselle	48° 59' N	7° 30' E	brook	3 Sep 2009	13	0	0%	-
	Nied	Aube	Lorraine	Moselle	49° 01' N	6° 20' E	brook	4 Sep 2009	10	1	**10%**	A3
	pond de la Sgreg	St. Nabord	Lorraine	Vosges	48° 03' N	6° 36' E	pond	15 Aug 2009	5	1	**20%**	A2
	Saône	Vioménil	Lorraine	Vosges	48° 05' N	6° 10' E	brook	26 Aug 2009	5	0	0%	-
	Ruisseau des Noires Faignes	Aneuménil	Lorraine	Vosges	48° 06' N	6° 32' E	brook	25 Aug 2009	15	5	**33%**	A2(2), A3(3)
	Meurthe	St. Michel sur Meurthe	Lorraine	Vosges	48° 19' N	6° 55' E	river	26 Aug 2009	12	0	0%	-
	Ezrule	Chaumont-sur-Aire	Meuse	Meuse	48° 56' N	5° 15' E	brook	1 Oct 2009	5	2	**40%**	A2(1), A3(1)
	Orne	Ornel	Meuse	Meuse	49° 15' N	5° 37' E	brook	24 Sep 2009	15	8	**53%**	A2
	Thoré	St. Amans-Soult	Midi-Pyrénées	Tarn	43° 29' N	2° 29' E	river	1 Oct 2009	9	3	**33%**	A2(2), A3(1)
	Miosson	Bertandinière, Smarves	Poitou-Charentes	Vienne	46° 31' N	0° 22' E	brook	18 Jun 2010	15	3	**20%**	A2(1), A3(1), A4(1)
	Grozon	St. Barthélémy-Grozon	Rhône-Alpes	Ardèche	44° 59' N	4° 37' E	brook	27 Aug 2009	4	0	0%	-
	lac Léman (lake Geneva)	Thonon-les-Bains	Rhône-Alpes	Haute-Savoie	46° 23' N	6° 29' E	lake	Jul 2009	16	5	**31%**	A2(4), A3(1)
	lac Laffrey	La Bergogne	Rhône-Alpes	Isère	45° 00' N	5° 47' E	lake	Sep 2009	13	1	**8%**	A2
	Charpasonne	Panissières	Rhône-Alpes	Loire	45° 47' N	4° 20' E	brook	2 Jul 2009	14	10	**71%**	A2(3), A3(6), A4(1)
	Aix	Grézolles	Rhône-Alpes	Loire	45° 51' N	3° 57' E	brook	26 Aug 2008	20	8	**40%**	A2(3), A3(5)
	Azergues	Ternand	Rhône-Alpes	Rhône	45° 57' N	4° 32' E	river	3 Oct 2009	3	0	0%	-
other species												
P. clarkii	La Chaume	Rosnay	Indre	Centre	46° 42' N	1° 13' E	pond	4 Mar 2011	2	1	**50%**	A2
O. limosus	pond Barineau	Rosnay	Indre	Centre	46° 42' N	1° 13' E	pond	4 Mar 2011	3	0	0%	-
O. limosus	Ramiers	Vernoux en Vivarais	Rhône-Alpes	Ardèche	44° 54' N	4° 39' E	brook	19 May 2009	16	0	0%	-
O. immunis	Reipertswiller	Rothbach	Alsace	Bas-Rhin	48° 56' N	7° 30' E	brook	2010	7	2	**29%**	A2

Discussion

Our results provide the first insight into the prevalence of the crayfish plague pathogen in invasive crayfish in France. We confirm that signal crayfish *Pacifastacus leniusculus* serves as an important reservoir of this disease in France, which supports previous suggestions of the key role of this invasive species in mortalities of native crayfish.

Our study shows that 20% of analysed signal crayfish from France were infected by the crayfish plague, with infected individuals present in 24 out of 45 studied populations. Similar percentage (21%) of infected signal crayfish was also detected by Kozubíková et al. (2011) who showed that in Central European populations of this species (from the Czech Republic, Slovakia, and Hungary), 32 out of 153 tested individuals were infected, coming from 8 out of 9 tested populations. In the one population from Norway analysed by Vrålstad et al. (2011), 38 out of 44 signal crayfish (i.e., 86%) were infected; such high prevalence within a population was only rarely found in France. In contrast, no traces of the pathogen DNA were detected in 44 individuals from the river Alling Å in Denmark (Skov et al. 2009). In another invasive crayfish *Orconectes limosus*, the number of infected individuals was found to be generally higher than in signal crayfish. In *O. limosus* from Central Europe, 116 out of 307 tested individuals (38%) were infected, coming from 16 out of 20 analysed populations (Kozubíková et al. 2011).

Nevertheless, the interpretation of present results should be careful as the real *A. astaci* prevalence observed in our study may be underestimated due to the fact that only a small number of individuals were tested in some populations. Moreover, only certain parts of the crayfish cuticle (from abdomen and uropod) were analysed and just a part of DNA isolate was used in the real-time PCR, which might also contribute to underestimations. However, as Vrålstad et al. (2011) found uropod tissue to be generally more infected than other parts of the crayfish body, we assume that some pathogen DNA should have been present in samples from most infected crayfish individuals analysed in our study. Other characteristics and limits of this method (such as its specificity against other closely related species of *Aphanomyces*, or its sensitivity) are discussed by Kozubíková et al. (2011) and Tuffs and Oidtmann (2011). Temporal fluctuations of the pathogen prevalence (or detectability) could also influence our results, as was shown for a Czech *Orconectes limosus* population studied over several seasons (Matasová et al. 2011). Thus, low *A. astaci* prevalence detected in some French signal crayfish populations does not mean these populations are less dangerous than other more infected populations.

In France, some regions seem to have more infected populations (Limousin, Rhône-Alpes, Basse-Normandie, Meuse or Midi-Pyrénées), while in others relatively low prevalence of the crayfish plague pathogen was found (Lorraine, Champagne-Ardenne or Languedoc-

Rousillon) (Table 1, Fig. 2). One of the localities which deserves particular attention is Lake Geneva (Lac Léman), where over 31% of tested *P. leniusculus* individuals were infected by *A. astaci*. Signal crayfish is intensively harvested there: the total yield per year 2001 was more than 56 metric tons of crayfish captured on the 30-km long French shore (Dubois et al. 2003). Although the French law does not permit transport and selling of invasive species, special prefectoral regulation allows fishermen to sell crayfish from Lake Geneva anywhere in France. They may therefore be exported alive to the rest of the country under a label "crayfish from Lac Léman" (Collas et al. 2007, Dubois et al. 2003). Such commercial activities may contribute to spreading of crayfish plague. Indeed, one French aquaculture company who offers *P. leniusculus* from lake Geneva sells also *A. leptodactylus* which do not seem to live long after the purchase (T. Duperray, pers. comm.). More measures should therefore be done to avoid further spread of signal crayfish from this lake to other parts of France or elsewhere.

However, Lake Geneva is also interesting by the evidence for extended coexistence between American and European crayfish species. The signal crayfish has been established in the lake for a long time, already since 1976 (Dubois et al. 2003). However, the narrow-clawed crayfish *Astacus leptodactylus* (a European species not indigenous to this region) was found in the lake at least until 2001 (Dubois et al. 2003) and it seems that the native white-clawed crayfish *Austropotamobius pallipes* was also present there at least until 2003 (C. Bugnon, pers. comm.). Several cases of a long-term coexistence between native species and *P. leniusculus* have also been recorded in other European countries. In England, *A. pallipes* coexisted with signal crayfish for more than 5 years (Souty-Grosset et al. 2006). In Finland, Westman and Savolainen (2001) observed a 30-year coexistence of *P. leniusculus* with another native species, *Astacus astacus*, although the latter was finally outcompeted. In these cases, it has been suggested that the invasive species was not infected by crayfish plague. However, Kozubíková et al. (2011) showed that in the Czech Republic, native noble crayfish *Astacus astacus* coexisted for at least ten years with *P. leniusculus* population, in which a low agent level (A2) of *A. astaci* was recently found in two out of 23 analysed individuals. In our study, five out of 16 signal crayfish from the lake Geneva were infected, with agent level A3 detected in one individual and A2 in four individuals (Table 1).

It is probable that the signal crayfish as well as the spiny-cheek crayfish *O. limosus*, also present in the lake, have contributed to the decline of susceptible species *Astacus leptodactylus* and *A. pallipes* at this locality. No information on crayfish plague outbreaks in the lake exist, we cannot therefore assess if this decline was caused by plague outbreaks, competition with invasive crayfish, other reasons, or a combination of multiple factors. Nevertheless, recent studies show that the coexistence of native European crayfish with the

crayfish plague pathogen is possible for long periods, as was shown in Turkey, Romania and Finland (Jussila et al. 2011, Pârvulescu et al. 2012, Svoboda et al. 2012, Viljamaa-Dirks et al. 2011). Thus, the presence of the pathogen in the environment does not necessarily lead to a complete disappearance of the native species at the locality. Mechanisms allowing such coexistence are not clear, but some authors suggest that water chemistry, increased resistance of European species to the pathogen, or reduced virulence of some *A. astaci* strains may have played a role (e.g. Harlioğlu 2008, Jussila et al. 2011, Svoboda et al. 2012, Viljamaa-Dirks et al. 2011).

In some French regions, the native *Austropotamobius pallipes* is still relatively abundant (e.g., in Ardèche department with 57 sites of *A. pallipes* recorded in 1998), while in others it is very rare or even absent (Marne department) (Machino and Diéguez-Uribeondo 1998). Main centres of its distribution are in central and south-eastern France, the former being also occupied by numerous signal crayfish populations (Collas et al. 2007). High priority should therefore be given especially to the protection of those localities where *A. pallipes* is abundant but infected *P. leniusculus* populations are at proximity. Lately, mass mortalities of *A. pallipes* have been recorded in the Ardèche department, especially in the Grozon brook near St. Barthélémy-Grozon (T. Duperray, pers. comm.). Signal crayfish from this brook did not test positive for *A. astaci*, but only four individuals from the area were analysed in our study. However, Ardèche department is a part of Rhône-Alpes region, where infected signal crayfish were found in most of the sampled populations. Attention should therefore be focused to this area, and further analyses of the pathogen prevalence in local signal crayfish should follow. At another locality in the Rhône-Alpes region, population of *A. pallipes* has disappeared from a brook la Font d'Aix between 1998 and 2008 (T. Duperray, pers. comm.). The cause of the decline is unknown, but crayfish plague may have played its role, as 40% of the 20 analysed signal crayfish from a close population in Aix brook at Grézolles were infected by the pathogen (Table 1). In another region, Auvergne, crayfish plague caused massive mortalities of *A. pallipes* in La Cère brook in 2008 (T. Pantarotto, pers. comm.). Our results show that 20% of signal crayfish from this brook at Sansac de Marmiesse were infected by *Aphanomyces astaci*. In Haute-Normandie, over 8% of analysed signal crayfish from a brook de la Côte Saint-Gilles (Saint Aubin sur Gaillon) were infected and therefore represent a threat to a population of *A. pallipes* from a nearby brook, situated about 3 km away (L. Desormeaux, pers. comm.).

Eradication of *P. leniusculus* from large areas is not possible so far (Souty-Grosset et al. 2006). However, our results can contribute to a development of an efficient strategy of conservation management of native crayfish in France. More attention should be paid to areas where high prevalence of crayfish plague in tested *P. leniusculus* population was found, as these represent higher danger for native species (Oidtmann et al. 2006).

The fact that we found infected individuals in more than a half of the studied populations confirms that the signal crayfish plays an important role in the transmission of the crayfish plague pathogen in France, and that it represents a serious threat to native crayfish, especially to the endangered *Austropotamobius pallipes*. Our analysis also shows that other non-indigenous crayfish species, including the "new" invader *Orconectes immunis,* may serve as sources of the disease in France. Further studies of the crayfish plague prevalence in other non-indigenous species would therefore be also important for evaluation of the risk those species represent for native crayfish.

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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.

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Re-examination of the prevalence of *Aphanomyces astaci* in North American crayfish populations in Central Europe by TaqMan MGB real-time PCR

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ABSTRACT: We applied quantitative TaqMan minor groove binder real-time polymerase chain reaction (PCR) on DNA isolates from soft abdominal cuticle of 460 North American crayfish *Orconectes limosus* and *Pacifastacus leniusculus*, previously tested for *Aphanomyces astaci* presence by conventional semi-nested PCR. Both approaches target the internal transcribed spacers of the pathogen nuclear ribosomal DNA, but apply different specific sequence motifs and technologies. The real-time PCR approach seems to provide higher sensitivity; the number of crayfish that tested positive increased from 23 to 32%, and 10 additional crayfish populations were indicated as hosting the disease agent. However, the vast majority of newly recorded positives contained very low agent levels, from 5 to 50 PCR-forming units. An isolate producing a false positive result by the semi-nested PCR (apparently undescribed *Aphanomyces* related to *A. astaci*) remained negative using the real-time PCR. The present study shows that previous results based on the semi-nested PCR were not substantially influenced by false positives but might have suffered from some false negatives at low agent levels. Combining alternative methods may therefore provide more reliable conclusions on the pathogen's presence. Further, we found positive correlation between the prevalence of infection carriers in American crayfish populations and the average amounts of *A. astaci* DNA detected in infected local crayfish individuals.

KEY WORDS: Crayfish plague · *Aphanomyces astaci* · *Pacifastacus leniusculus* · *Orconectes limosus* · Semi-nested PCR · Real-time PCR · Pathogen detection · Agent level

INTRODUCTION

The crayfish plague, i.e. mass mortalities of indigenous European crayfish caused by the oomycete *Aphanomyces astaci* (Saprolegniaceae), has serious conservational as well as economical consequences (Holdich et al. 2009) and requires fast and reliable diagnostics. Several alternative PCR-based assays for detection of the crayfish plague pathogen have been described (Oidtmann et al. 2004, 2006, Hochwimmer et al. 2009, Vrålstad et al. 2009). The first molecular method for *A. astaci* detection from clinical samples (based on internal transcribed spacer [ITS] in the nuclear ribosomal DNA; Oidtmann et al. 2004) considerably sped up and improved the reliability of parasite diagnostics. However, the method provided insufficient specificity against the closely related *A. frigidophilus* and *A. invadans*

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(Oidtmann et al. 2006), the first of which was isolated from crayfish as well (Ballesteros et al. 2006).

Therefore, Oidtmann et al. (2006) improved the assay by developing a new forward PCR primer that discriminated against these closely related species. For detection of the agent in North American (hereafter 'American') carrier species (including spiny-cheek crayfish *Orconectes limosus* and signal crayfish *Pacifastacus leniusculus*) 2 PCR protocols (one single-round and one semi-nested PCR) were established; these were sensitive enough to detect *Aphanomyces astaci* in symptom-free carrier crayfish. Both protocols reliably detected the crayfish plague pathogen, but the semi-nested PCR also reacted to extremely high concentrations of *A. frigidophilus* and *A. invadans* DNA. For this reason, Oidtmann et al. (2006) recommended the single-round PCR (not sensitive to the above-mentioned species) combined with sequencing for confirmation of *A. astaci* detection (OIE 2010). The product of the single-round PCR is suitable for distinguishing *A. astaci* from other oomycetes, as the sequence of the resulting ITS fragment is nearly invariable in all known *A. astaci* strains, but clearly different even from the most related known species (Diéguez-Uribeondo et al. 2009, Takuma et al. 2010, Makkonen et al. 2011). Sequencing of PCR products after single-round PCR has so far resulted in only one discovery of a false positive result (Diéguez-Uribeondo et al. 2009, Kozubíková et al. 2009), demonstrating the amplification of DNA from an hitherto unknown *Aphanomyces* lineage closely related to *A. astaci* (GenBank acc. no. FM955258) from a signal crayfish. However, this oomycete strain has not been isolated to a laboratory culture, preventing further studies and a formal description. The single-round assay according to Oidtmann et al. (2006) thus remains a very reliable method for the detection of *A. astaci*, very rare errors of which may be uncovered with sequencing, and is officially recommended by the World Organisation for Animal Health (OIE 2010).

An alternative protocol using quantitative TaqMan real-time PCR (Vrålstad et al. 2009) targets a variable part of the ITS1 region specific to *Aphanomyces astaci* using 2 specific primers and 1 specific TaqMan minor groove binder (MGB) probe. This probe provides higher stringency, and consequently increased specificity, than conventional primers (Vrålstad et al. 2009). Additionally, the real-time PCR approach provides lower risk of laboratory-induced contamination (there is no further manipulation of PCR products after the reaction), increased sensitivity of agent detection, and quantitative results. However, a disadvantage of the real-time PCR assay is that its PCR product is not suitable for sequencing; thus, confirmation of the identity of the amplified fragment is not possible without conventional PCR. Simultaneously, another real-time PCR assay based on the detection of the gene for endochitinase was developed (Hochwimmer et al. 2009).

All molecular methods mentioned above have been tested against the DNA of various oomycete cultures, but published tests of DNA samples isolated directly from crayfish tissues are limited. These methods have already been used several times to answer questions concerning the distribution and prevalence of *Aphanomyces astaci* in invasive American crayfish populations in Europe (e.g. Schulz et al. 2006, Aquiloni et al. 2011, Skov et al. 2011); however, in most cases, only a few populations were analysed. The only published extensive study on the prevalence of *A. astaci* in American crayfish populations on a national scale (Kozubíková et al. 2009) was based on the semi-nested PCR by Oidtmann et al. (2006). Since Oidtmann et al. (2006) recommended using a single-round PCR protocol combined with sequencing, we also later applied this method to samples that tested positive in the semi-nested PCR; 80% of those samples showed positive results in the single-round PCR, suggesting that the semi-nested PCR was more sensitive (E. Kozubíková unpubl. data). However, alternative explanations of this observation could be that some proportion of samples positive in the semi-nested PCR were actually false positives, or that the DNA isolates became degraded by long-term storage (Oidtmann et al. 2006). To rule out the possibility that the semi-nested PCR protocol suffers from false positive results when applied to field samples, we decided to verify our previous results by an alternative method that has recently become available.

The development of specific assays for *Aphanomyces astaci* detection is an on-going process, because our knowledge about the diversity of related species possibly cross-reacting with the existing methods is still deficient. A combination of available methods may therefore improve the reliability of results. In the present study, we re-examined previously analysed samples originating from 3 Central European countries (Czech Republic, Hungary, and Slovakia) by quantitative TaqMan-MGB real-time PCR (Vrålstad et al. 2009) in order to (1) evaluate whether the previous results from the semi-nested PCR have been significantly influenced by false positives, (2) test whether the use of a different ITS-based detection method substantially influences the

general patterns of known distribution of *A. astaci* in invasive crayfish populations, and (3) obtain semi-quantitative data on the level of agent DNA in samples for comparison to the *A. astaci* prevalence in the carrier populations of American crayfish.

MATERIALS AND METHODS

DNA isolates

For the present study, we used 460 DNA isolates from soft abdominal cuticles of spiny-cheek crayfish *Orconectes limosus* (307 samples) and signal crayfish *Pacifastacus leniusculus* (153 samples). The crayfish came from 25 populations in the Czech Republic, 3 in Hungary and 1 in Slovakia. Each sample represented 1 crayfish individual. The samples were originally obtained for Kozubíková et al. (2006, 2008, 2009, 2010) and Petrusek & Petrusková (2007) (see Table 2 for a list of localities and the number of analysed individuals). DNA was isolated using the DNeasy tissue kit (Qiagen) from soft abdominal cuticle (in individuals smaller than 5 cm a part of an uropod or telson was also included) as described by Kozubíková et al. (2009), and the isolates were stored for 1 to 5 yr in –20°C. We also included a DNA isolate from a signal crayfish individual from the Czech Republic that tested false positive by conventional PCR methods (both the semi-nested and the single-round assays), but was proven to be a different *Aphanomyces* lineage by sequencing the PCR product of the single-round PCR (GenBank acc. no. FM955258; Kozubíková et al. 2009). ITS sequences were also available for 14 samples that were confirmed in that way to contain *Aphanomyces astaci* DNA (Diéguez-Uribeondo et al. 2009, Kozubíková et al. 2009). All these sequences (including those submitted to GenBank under acc. nos. FM999252 to FM999259 and FM999239) were invariable.

Semi-nested PCR

All DNA isolates were analysed for *Aphanomyces astaci* presence by semi-nested PCR; 15 samples were specifically amplified for the purpose of the present study. Results for the remaining 445 samples were published by Kozubíková et al. (2006, 2008, 2009, 2010). All samples were processed as described by Kozubíková et al. (2009). The primers '42' and '640' (first PCR run) and '525' and '640' (second PCR run, using the product of the first PCR as a template) after Oidtmann et al. (2006) were used to amplify an *A. astaci*–diagnostic fragment of rDNA. Each 50 µl PCR reaction contained 1.25 U of *Taq* DNA polymerase, 1× *Taq* buffer (with KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (reagents from Fermentas), 0.5 µM of each primer, and 10 µl of template DNA. Cycling conditions followed the protocol provided by Oidtmann et al. (2006). After agarose electrophoresis, a DNA fragment identical in length to that obtained from the positive control (DNA isolate from a clean laboratory culture of *A. astaci* of the Strain M96/1, Genetic Group B from Oidtmann et al. 1999) was considered to show detection of *A. astaci* in the individual crayfish.

TaqMan MGB real-time PCR

Quantitative detection of *Aphanomyces astaci* by real-time PCR was performed as described by Vrålstad et al. (2009) using an *A. astaci*-specific pair of primers (AphAstITS-39F and AphAstITS-97R) combined with the *A. astaci*-specific MGB probe (AphAstITS-60P). The total reaction volume of 25 µl contained 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems) with the passive reference dye ROX, 500 nM of the forward and reverse primers, 200 nM of the MGB probe, 1.5 µl sterile milliQ water, and 5 µl of template DNA. Amplification and detection were performed on the Mx3005P qPCR system (Stratagene) whereby the PCR reactions were set up in 96-well polypropylene plates sealed with 8× strip optical caps for Stratagene (Agilent Technologies). The PCR program included an initial decontamination step of 2 min at 50°C followed by 10 min at 95°C for DNA polymerase activation, uracil N-glycosylase deactivation and template DNA denaturation. Afterwards, 50 cycles of 15 s at 95°C and 60 s at 58°C were carried out. Four calibration points of a standard series of known PFU (PCR-forming units or amplifiable copies of the target DNA) content were included in each run (see Vrålstad et al. 2009). In order to avoid carry-over contamination from the standard, the 4 calibrants were added to the plate after sealing all other unknown crayfish DNA samples with the 8× strip caps. Finally, negative PCR controls were included in all runs; these remained negative in all cases.

The sample that included the DNA of the new *Aphanomyces* lineage related to *A. astaci* (FM955258; Kozubíková et al. 2009) was tested further with the TaqMan Environmental Master Mix (Applied Biosystems), which appears to work more effectively ac-

cording to Strand et al. (2011). Two separate tests (both repeated twice) were performed: (1) under the same conditions as described above and (2) with elevated annealing and synthesis temperature using 50 cycles of 15 s at 95°C and 60 s at 60°C. Only the 10× diluted sample was available for these additional tests, as the stock of the DNA isolate became limited.

Quantification of real-time PCR data

The data were analysed in the MxPro software V.4.10 (Stratagene). The calibration points were used to generate a standard curve for quantification of *Aphanomyces astaci* in terms of PFU in the unknown crayfish tissue samples, and corresponded to the standards 1, 3, 7, and 9 in Vrålstad et al. (2009), with estimated numbers of PFU corresponding to 3×4^{10}, 3×4^{8}, 3×4^{4}, and 3×4^{2} (Table 3 in Vrålstad et al. 2009). Each DNA isolate was tested with an undiluted and a 10-fold diluted replicate.

Absolute quantification is possible in the absence of PCR inhibition above the limit of quantification (LOQ = 50 PFU; Vrålstad et al. 2009). The presence or absence of real-time PCR inhibition was controlled by calculating the difference in Ct (cycle threshold) values (ΔCt) between the undiluted and corresponding 10-fold diluted DNA replicates. In the absence of inhibition, ideal amplification efficiency and no laboratory-induced inaccuracies, the theoretical ΔCt value equals 3.32. In practice, some variation arises due to minor inaccuracies related to amplification efficiency, manual pipetting, and other stochastic factors. Here, we considered acceptable a variance level of 15%, then allowing for quantification in samples where the ΔCt is 3.32 ± 0.5 (range = 2.82 to 3.82) between the undiluted and 10-fold diluted replicates. For samples where *Aphanomyces astaci* could be quantified above LOQ, the final PFU-values were estimated as follows: when ΔCt was within the accepted range from 2.82 to 3.83, the final PFU-value was calculated as the mean of the undiluted PFU-value and the 10-fold diluted PFU-value, the latter multiplied by 10. If ΔCt was <2.82 (indicating inhibition) or >3.82 (i.e. 10-fold dilution out of range), the final PFU-values could not be calculated accurately, but for comparative purposes we used an estimate based on the most relevant of the 2 values obtained. In the former case, the estimated PFU was based on the 10-fold diluted DNA replicate alone (for which the effects of inhibition were expected to be eliminated or less pronounced), and, in the latter case, it was based solely on the undiluted DNA replicate.

Finally, all samples were assigned to the more comprehensible semi-quantitative categories (agent levels) suggested by Vrålstad et al. (2009). These categories and their limit values are described in Table 1.

Statistical analyses

The proportion of crayfish individuals that tested positive with the 2 methods was compared by the Chi-squared test, for all analysed individuals pooled as well as separately for the 2 crayfish species. The relationship between the prevalence of infected individuals in the crayfish populations (i.e. the likelihood that any particular crayfish in the population sample tests positive by real-time PCR) and the average amount of the pathogen DNA detected in apparently infected crayfish individuals from each population (expressed as PFU-values; log-transformed for the analysis) was analysed separately for both host crayfish species by logistic regression (using the maximum-likelihood loss function and quasi-Newton estimation method). For the calculation of the average pathogen load, we also included crayfish individuals with Agent Level A2. Although these levels of the pathogen DNA are below the limit of reliable quantification, we used the resulting low (though less accurate) PFU-values in the calculation in order to avoid an artificial increase in the estimated average load, as would be expected if the lightly infected crayfish hosts were excluded. The tests were performed in the software Statistica V.6.1 (StatSoft).

RESULTS

Prevalence of *Aphanomyces astaci* carriers in crayfish populations: re-evaluation of previous data

The results of the prevalence of *Aphanomyces astaci* in American crayfish *Orconectes limosus* and *Pacifastacus leniusculus* obtained by the semi-nested PCR and the real-time PCR are summarised in Table 2 according to crayfish species and origin. The real-time PCR approach detected the pathogen in 46 crayfish individuals that remained negative with the semi-nested PCR (Table 3, Fig. 1a). With 5 exceptions, these detections represented Agent Level A2 (Table 3). The remaining 5 semi-nested PCR negative results were detected at Agent Level A3, but the PFU-values were <100 in all cases.

Table 1. Semi-quantitative categories of agent levels of *Aphanomyces astaci* in a test sample based on the number of PCR-forming units (PFU) detected (after Vrålstad et al. 2009). Agent Level A1 (traces below the limit of detection) may indicate a minute or very early sign of infection, but could also represent false positives in terms of PCR artifacts or minimal carry-over contamination from another sample; such a result is consequently not taken as sufficient evidence for the pathogen's detection. PFU refers to amplifiable DNA copies of the analyte (the 57 bp DNA-sequence motif of *A. astaci*) in a PCR reaction tube. Ct (cycle threshold) values are based on the study by Vrålstad et al. (2009). Differences in the signal acquisition systems will result in minor differences in measured Ct-values with different thermal cyclers; values listed here are thus only guiding and not absolute. A molecular assay must always be validated in-house and calibrated based on the assigned concentrations of the standards (calibration material) prior to application in diagnostics on a new thermal cycler or in a new laboratory. LOD: limit of detection (defined as 95% probability of detection: 5 PFU); LOQ: limit of quantification (50 PFU); obs: observed values

Agent level	PFU in sample	Expected Ct	Result	Interpretation
A0	0 or below Ct cut-off value	Undetermined or Ct > 41	Not detected	Negative
A1	Detected below LOD (PFU_{obs} < 5 PFU)	41 ≥ Ct_{obs} > 39	Detected below LOD	Trace amounts, not a reliable detection
A2	LOD ≤ PFU_{obs} < LOQ = 50 PFU	39 ≥ Ct_{obs} > 34.7	Detected	Very low levels of *A. astaci* DNA in sample (below LOQ)
A3	LOQ ≤ PFU_{obs} < 10^3 PFU	34.7 ≥ Ct_{obs} > 30.0	Detected	Low levels of *A. astaci* DNA in sample
A4	10^3 PFU ≤ PFU_{obs} < 10^4 PFU	30.0 ≥ Ct_{obs} > 26.2	Detected	Moderate levels of *A. astaci* DNA in sample
A5	10^4 PFU ≤ PFU_{obs} < 10^5 PFU	26.2 ≥ Ct_{obs} > 22.6	Detected	High levels of *A. astaci* DNA in sample
A6	10^5 PFU ≤ PFU_{obs} < 10^6 PFU	22.6 ≥ Ct_{obs} > 18.5	Detected	Very high levels of *A. astaci* DNA in sample
A7	10^6 PFU ≤ PFU_{obs}	Ct_{obs} ≤ 18.5	Detected	Exceptionally high levels of *A. astaci* DNA in sample

The percentage of samples testing positive (excluding the 1 known false positive reported by Kozubíková et al. 2009) significantly increased when the real-time PCR rather than the semi-nested PCR was used ($\chi^2 = 8.7$, $p = 0.003$): from 23 to 32% (Fig. 1a, Table 2). Interestingly, the majority of new detections were from signal crayfish, where the prevalence of *Aphanomyces astaci* increased from 3% of all analysed individuals to 21%. This increase was highly significant ($\chi^2 = 22.4$, $p < 0.0001$), which was not the case for spiny-cheek crayfish. In the latter species, the proportion of individuals that tested positive increased from 34 to 38% ($\chi^2 = 1.2$, $p = 0.27$).

Overall, the number of crayfish populations with 1 or more individual with a positive test among the examined individuals increased from 14 with the semi-nested PCR to 24 with the real-time PCR (Table 2). For the remaining 5 of the 29 tested populations where no infected individual was detected, only a low number of individuals were tested (11 from a Köszeg boating pond in Hungary, otherwise 1 to 3 individuals). These negative results are therefore of no conclusive value. Two individuals with very low levels of the target DNA (Agent Level A2) were discovered in a population of signal crayfish that had coexisted with the native European species *Astacus astacus* for at least 10 yr.

The semi-nested PCR versus the quantitative TaqMan real-time PCR

The real-time PCR detected the target DNA in a considerably higher number of samples than did the semi-nested PCR (Table 3, Fig. 1a). Although the latter method was less sensitive, it worked with 100% reliability in samples containing >100 PFU per reaction quantified with the real-time PCR. The efficiency of the semi-nested PCR compared to the real-time PCR was 92% in the category A3, but only 41% in the category A2 (Table 3).

Table 3 shows that all but 7 samples (i.e. 94%) that tested positive for *Aphanomyces astaci* using the semi-nested PCR also produced a positive result above the limit of detection (LOD) with the real-time PCR. The 7 samples not confirmed as positive with the real-time PCR included 5 samples in which trace amounts of the target DNA were detected below LOD (Agent Level A1). In the remaining 2 DNA isolates, no Ct or Ct >41 (the cut-off value, see Table 1) was detected (Agent Level A0). One of these was an already known case of false positive detection, an isolate containing the DNA of a putative undescribed *Aphanomyces* sp. closely related to *A. astaci* from signal crayfish from the Czech Republic (GenBank acc. no. FM955258; Kozubíková

Table 2. Detailed results of *Aphanomyces astaci* prevalence in North American crayfish *Orconectes limosus* and *Pacifastacus leniusculus* populations obtained by semi-nested PCR and real-time PCR assays. Semi-nested PCR results were published by Kozubíková et al. (2006, 2008, 2009, 2010). Results for samples originating in the Czech Republic are summarised separately to allow direct comparison with Kozubíková et al. (2009). A5 to A2: agent levels (see Table 1)

Crayfish sampling locality	Animals tested	Semi-nested PCR		Real-time PCR					
		No. positive	%	A5	A4	A3	A2	No. positive	%
Orconectes limosus									
Jickovický Brook (49° 26′ N, 14° 13′ E)	13	13	100		1	12		13	100
Prudník Brook (50° 17′ N, 17° 43′ E)	11	11	100		1	10		11	100
Smečno village pond (50° 11′ N, 14° 02′ E)	40	39	98	1	10	21	7	39	98
Kořensko Reservoir (49° 14′ N, 14° 22′ E)	3	2	67		2			2	67
Elbe River (5 different sites)[a]	20	12	60		1	5	6	12	60
Pšovka Brook (50° 23′ N, 14° 33′ E)	18	9	50			6	5	11	61
Zlatá stoka Brook (49° 00′ N, 14° 46′ E)[a]	19	8	42				5	5	26
Hracholusky Reservoir (49° 47′ N, 13° 07′ E)[a]	20	4	20				3	3	15
Malše River (48° 57′ N, 14° 28′ E)	12	1	8			2	1	3	25
Proboštská jezera Lake (50° 12′ N, 14° 39′ E)	17	1	6			1	5	6	35
Klíčov flooded quarry (49° 24′ N, 12° 57′ E)[a]	40	1	3				1	1	3
Kojetice flooded sandpit (50° 14′ N, 14° 31′ E)	20		0				3	3	15
Cítov flooded sandpit (50° 24′ N, 14° 23′ E)	10		0				2	2	20
Lhota flooded sandpit (50° 14′ N, 14° 40′ E)	33		0				2	2	6
Kamenička Brook (50° 44′ N, 14° 11′ E)	1		0				1	1	100
Barbora flooded coal mine (50° 38′ N, 13° 45′ E)	2		0						0
Cidlina River (50° 07′ N, 15° 10′ E)	3		0						0
Račice flooded sandpit (50° 26′ N, 14° 19′ E)	2		0						0
Vltava River (50° 08′ N, 14° 23′ E)	1		0						0
Summary—Czech populations	285	101	35					114	40
Danube, Hungary (46° 21′ N, 18° 53′ E)[b]	22	2	9			1	1	2	9
Summary—*O. limosus*	307	103	34	1	15	58	42	116	38
Pacifastacus leniusculus									
Stržek fishpond (49° 22′ N, 16° 04′ E)	20	1	5				2	2	10
Ráček II fishpond (49° 39′ N, 16° 18′ E)	23	1[c]	4				2	2	9
Nad tratí fishpond (49° 22′ N, 16° 04′ E)	49		0			3	15	18	37
Blanice River (49° 09′ N, 14° 10′ E)	8		0				2	2	25
Spustík fishpond (49° 22′ N, 16° 07′ E)	13		0				2	2	15
Kouba Brook (49° 19′ N, 13° 01′ E)	11		0				1	1	9
Summary—Czech populations	124	1[d]	1					27	22
Gyöngyös River, Hungary (47° 23′ N, 16° 32′ E)[b]	16	4	25			2	2	4	25
Köszeg Pond, Hungary (47° 23′ N, 16° 32′ E)[b]	11		0						0
Morava River, Slovakia (48° 24′ N, 16° 51′ E)	2		0				1	1	50
Summary—*P. leniusculus*	153	5[d]	3			5	27	32	21
Summary—both species	460	108[d]	23	1	15	63	69	148	32

[a]One to 3 samples from each of these populations were found to be positive with the semi-nested PCR but negative with the real-time PCR (putative false positives of the semi-nested PCR)
[b]Real-time PCR results published by Kozubíková et al. (2010)
[c]False positive result of the semi-nested PCR confirmed by sequencing
[d]False positive result from Ráček II fishpond was not included in summary values as it was not considered positive in the study by Kozubíková et al. (2009)

et al. 2009). A weak signal appeared in the tests using the TaqMan Universal PCR Master Mix, but never crossed the fixed baseline (=0.15). In the tests using the TaqMan Environmental Master Mix, the putative false positive was detected at Ct 42, well below the cut-off value. Further, with elevated annealing and synthesis temperatures, the signal touched the baseline at Ct 50 (Fig. 2).

Quantitative results

Among all samples tested in the present study, 68% were negative. A further 15% fell within Agent Level A2 (under the LOQ), in which the undiluted DNA was detected in the A2 category, while the 10-fold diluted DNA remained negative or was detected in the A1 category. The remaining 17% of the sam-

Table 3. Efficiency of the semi-nested PCR assay compared to the real-time PCR for detection of *Aphanomyces astaci*. Several cases of *A. astaci* detections were confirmed by sequencing at each agent level (see Table 1). Sequence results are from Kozubíková et al. (2009); accession numbers of those submitted to GenBank are FM999239 and FM999252 to FM999259, the 5 remaining sequences were invariable. Sequenced PCR products represented various host populations, and were not chosen according to agent level. LOQ: limit of quantification (50 PFU); LOD: limit of detection (defined as 95% probability of detection; 5 PFU); A0: all samples with no detection or with detection below the cycle threshold cut-off value of 41

Agent level	Real-time PCR (no. in each category)	Semi-nested PCR (no. positive)	%	No. of samples sequenced as *A. astaci*
A5	1	1	100	
A4	15	15	100	6
A3	63	58	92	4
A2 (detection below LOQ)	69	28	41	4
A1 (detection below LOD)	98	5		
A0 (negative)	214	2		

ples were detected above the LOQ, and fell into Agent Levels A3 (14%), A4 (3.3%), and A5 (0.2%). For these, quantitative PFU-values are listed in Table A1 in Appendix 1.

The quantitative PFU-values (based on medians with 10 and 90% percentiles) for each agent level above the LOQ are summarised in Table 4. For 68% of these samples, the obtained ΔCt value was within the range accepted for quantification. Severe inhibition was never observed, but 3.8% of the samples detected above the LOQ showed signs of minor inhibition (i.e. the observed ΔCt was slightly smaller than the values accepted for quantification purposes). Finally, in 28% of these samples, the 10-fold dilution was slightly out of range (i.e. the observed ΔCt was slightly larger than the values accepted for quantification). In the majority of these cases, the 10-fold diluted DNA replicate was diluted out of the quantitative range, and the undiluted replicate was detected in Agent Level A3 just above the LOQ (Table 4, Table A1).

The distribution of the real-time PCR results among the semi-quantitative agent levels for spiny-cheek crayfish and signal crayfish is summarised in Fig. 1b. For both species, almost 50% were negative, and 16 to 32% of the samples (for spiny-cheek crayfish and signal crayfish, respectively) fell into the A1 category (trace DNA amount, detection below LOD), which should not be regarded as reliable positives (Vrålstad et al. 2009). The remaining 38 and 21% of the samples (spiny-cheek crayfish and signal crayfish, respectively) were regarded as reliable positives (i.e. detection above LOD). The majority of positive samples of signal crayfish fell within Agent Level A2, while the majority of positive spiny-cheek crayfish samples fell within Agent Level A3. A small number of spiny-cheek crayfish fell within the A4 and A5 agent levels, while none of the analysed signal crayfish samples contained higher levels of agent DNA than A3 (Fig. 1b). Out of 14 samples from which the presence of *Aphanomyces astaci* DNA was confirmed by sequencing, 4 contained very low levels of

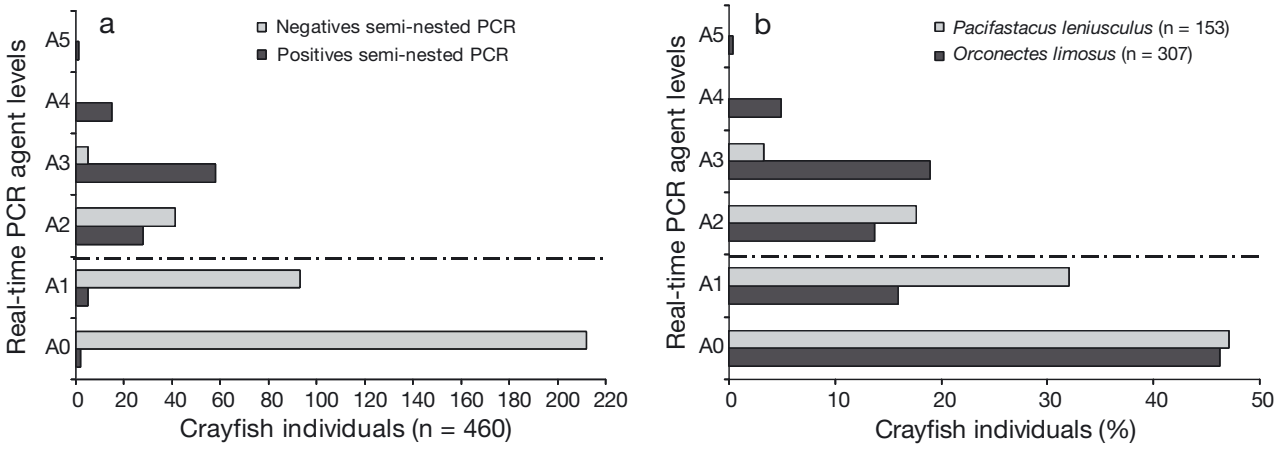

Fig. 1. (a) Numbers of positive and negative semi-nested PCR results compared to the corresponding real-time PCR results in terms of agent levels (defined in Table 1). (b) Summary of the real-time PCR results for *Orconectes limosus* and *Pacifastacus leniusculus*. The agent levels above the horizontal dashed line (A2 to A5) are considered to show reliable detection of *Aphanomyces astaci*

agent DNA (A2), while the remaining 10 samples fell within the categories A3 and A4 (Table 3).

The prevalence of *Aphanomyces astaci*-positive crayfish individuals per population positively correlated with the average levels of the parasite DNA detected in crayfish from each population (Fig. 3). Logistic regressions were significant for both host crayfish species (spiny-cheek crayfish: odds ratio per

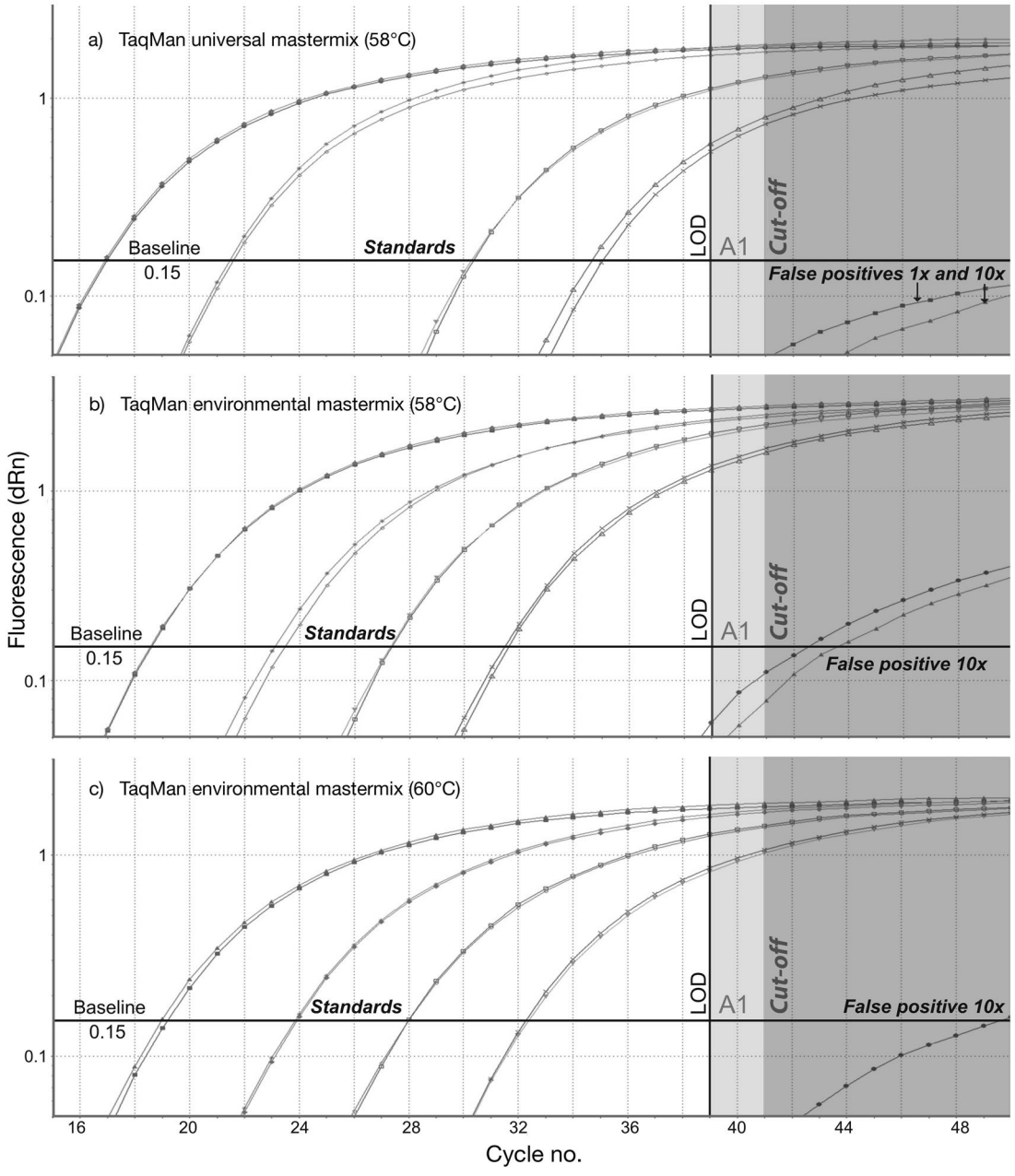

Fig. 2. Real-time PCR analyses of the sample containing DNA of *Aphanomyces* sp., which resulted in a false positive detection of the crayfish plague pathogen by conventional PCR (Genbank acc. no. FM955258; Kozubíková et al. 2009), performed with (a) TaqMan Universal PCR Master Mix and annealing and synthesis at 58°C, or (b,c) TaqMan® Environmental Master Mix and annealing and synthesis at (b) 58°C or (c) 60°C. The baseline was fixed at 0.15. The vertical black lines denote the limit of detection (LOD) of the real-time PCR assay (Ct 39). The dark grey area indicates the cut off area (Ct ≥41) in which any positive signals are excluded. The detection area for Agent Level A1 is indicated in light grey. The originally concentrated sample (1×) was no longer available for (b) and (c), for which only the 10-fold diluted original DNA was used. The standards in (a) and (b,c) correspond to standard numbers 1, 3, 7, and 9, and 2, 4, 6, and 8, respectively, from Vrålstad et al. (2009). The false positive DNA yields very weak signals that do not cross the baseline in (a), cross the baseline after Ct 42 in (b), and cross the baseline at Ct 50 in (c)

Table 4. Summary of quantitative samples. DNA samples detected above the limit of quantification (LOQ = 50 PFU). Samples in Agent Level A3 accounted for the majority (81%) of quantifiable samples (QS). PFU-values are based on median values with 10 and 90% percentiles in parentheses. Acceptable quantification (AQ): the difference in cycle threshold values (ΔCt) between the 10-fold diluted and the undiluted DNA replicates within the range of 3.32 ± 0.5. Non-acceptable quantification (NQ): a ΔCt > 3.82 indicates that the 10-fold diluted DNA replicate is out of range (OR). A ΔCt < 2.82 indicates minor inhibition (MI) in the undiluted DNA replicate. Background data are provided in Table A1

Agent level	Total QS (%)	Median PFU	Mean ΔCt	AQ (%)	NQ OR (%)	NQ MI (%)
A5	1.3	45695	3.47	100	0	0
A4	17.7	2000 (1259, 5815)	3.62 (±0.20)	86.7	13.3	0
A3	81	160 (69, 530)	3.76 (±0.78)	63.5	31.7	4.8
Overall	100			68.4	27.9	3.8

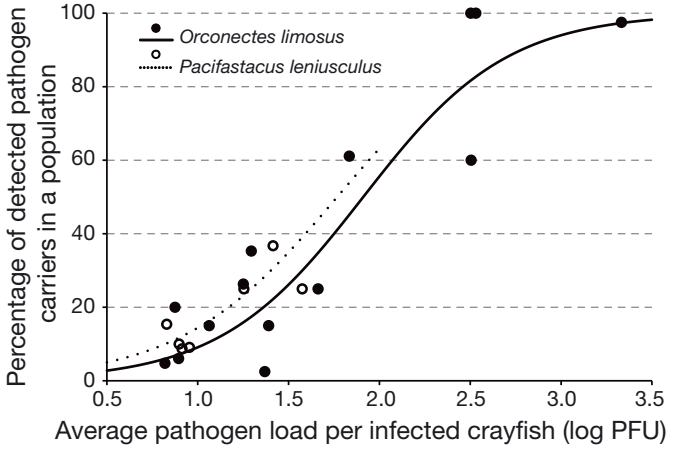

Fig. 3. Relationship between pathogen prevalence in the population and average agent level in infected individuals. The prevalence is estimated as a proportion of individuals testing positive for the presence of *Aphanomyces astaci*, the pathogen load (expressed as PFU detected in a real-time PCR reaction) is log-transformed for the analysis. Curves show the values predicted by logistic regressions: *Orconectes limosus*: $y = e^{-4.83+2.53x}/(1 + e^{-4.83+2.53x})$; *Pacifastacus leniusculus*: $y = e^{-4.10+2.32x}/(1 + e^{-4.10+2.32x})$

10-fold increase in PFU: 12.51, $\chi^2 = 160.8$, df = 1, $p < 10^{-7}$; signal crayfish: odds ratio: 10.19, $\chi^2 = 9.04$, df = 1, p = 0.0026). All samples containing $>10^3$ PFU per PCR reaction (Agent Levels A4 and A5) were found in populations with a high prevalence of infected crayfish individuals (60 to 100%; Table 2).

DISCUSSION

In the present study, the results of the semi-nested PCR (Kozubíková et al. 2009, 2010) were largely confirmed by real-time PCR in terms of positive carrier status and did not appear to be notably influenced by false positive results. In contrast, the real-time PCR assay significantly increased the overall number of crayfish that tested positive, suggesting that this method provides higher sensitivity. Using *Aphanomyces astaci* pure culture material and zoospores, Tuffs & Oidtmann (2011) demonstrated that the ITS real-time PCR assay is 10- and 100-fold more sensitive than conventional PCR (Oidtmann et al. 2006) and chitinase real-time PCR (Hochwimmer et al. 2009) assays, respectively. However, a comparative study of the ITS- and chitinase-based methods (Hochwimmer et al. 2009) used on crayfish samples is still lacking and might be useful.

Improvement of the molecular detection methods of *Aphanomyces astaci* is a continuous process. The recently discovered *A. salsuginosus* (Takuma et al. 2010) isolated from ice fish *Salangichthys microdon* in Asia is hitherto the closest described relative of *A. astaci* based on the ITS-sequence data and also resembles the *Aphanomyces* lineage (FM955258) yielding the false positive with conventional PCR methods (Fig. 4). These species, together with the assumed huge unknown diversity of oomycetes, are continuously challenging the claimed specificity of any *A. astaci* diagnostic methods. It is urgent to test real-time and conventional PCR methods against genuine DNA from *A. salsuginosus*. However, unlike conventional PCR assays, real-time PCR proved robust against the false positive *Aphanomyces* lineage, despite the high homology between this ITS sequence and the primer and probe motifs of the real-time assay (Fig. 4). Our test cannot exclude the possibility that the real-time assay could cross-react if higher concentrations of this false positive DNA were present in the reaction, but the observed robustness is probably a result of the high discriminatory ability of the MGB probe. In contrast to conventional TaqMan (hydrolysis) probes, MGB probe assays allow very little mismatch at the probing site (Yao et al. 2006). Interestingly, the observed signal delay with increased primer and probe annealing temperature indicates that an optimization of the method could further increase its robustness against false positives. These results also justify the use of cut-off values when interpreting real-time PCR results.

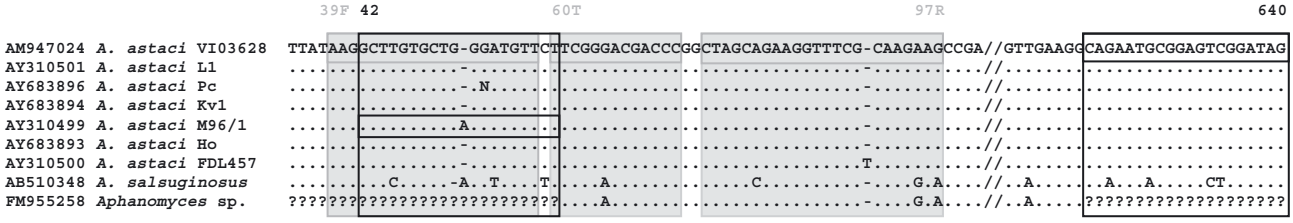

Fig. 4. Partial internal transcribed spacer sequence alignment reflecting the primer and probe sequence motifs of the real-time PCR (Vrålstad et al. 2009) and single-round conventional PCR (Oidtmann et al. 2006) assays for species-specific detection of *Aphanomyces astaci*. The solid gray boxes show the positions of the primers AphAstITS-39F and AphAstITS-97R, as well as the TaqMan MGB probe AphAstITS-60T. The open black boxes show the positions of the primers 42 and 640. Note that the primer 42 includes an insertion (extra A) only present in the *A. astaci* isolate ML96/1. The alignment is based on publicly available *A. astaci* sequences along with the sequence of the recently described *Aphanomyces salsuginosus* (Takuma et al. 2010) and the sequence obtained from the false positive detected by the primer pair 42/640 (*Aphanomyces* sp., FM955258; Kozubíková et al. 2009). The true sequence for the false positive is unknown in the region of primers 42 and 640 (denoted with question marks), since the sequence was amplified with these primers. The sequence motifs of *A. salsuginosus* and the false positive *Aphanomyces* sp. are largely overlapping in the regions of the primer AphAstITS-97R and the probe AphAstITS-60T

Apart from the confirmed false positive discussed above, only 6 additional samples (1.3%) were not reliably detected by the real-time PCR. Trace amounts of putative *Aphanomyces astaci* DNA were detected in 5 of those 6 samples (Agent Level A1; see Table 3). These may well represent false positives, taking the results above into account. Alternatively, they may reflect the phenomenon that the ITS target DNA copies are not evenly distributed in the DNA sample, as they occur in tandem rDNA repeats in the genome. If only a few such DNA strands are present in the original DNA extract, rapid depletion of the extract may occur, and only one or a few reactions will turn out to be positive. This will result in a positive/negative ratio corresponding to a probability of detection <95%, i.e. below LOD. Finally, the DNA samples in the present study had been stored for some years after DNA extraction, and the target DNA may have been partially degraded. Hence, it cannot be excluded that the 5 samples in question originally contained true traces of *A. astaci* DNA. Whatever the reason may be, it is important to maintain the A1 category in the real-time PCR procedure as uncertain and unreliable, as suggested by Vrålstad et al. (2009), since true traces of *A. astaci* DNA and false positive signals may well overlap within this category.

We detected new positives by real-time PCR only in the samples containing DNA levels around or below the LOD of the semi-nested PCR (Oidtmann et al. 2006). This assay seems to work reliably when >100 PFU enters the PCR reaction, which is above Agent Level A2. The semi-nested PCR can still detect the agent DNA in the A2 category, but not with 100% efficiency (Table 3), which agrees well with the validation tests of Tuffs & Oidtmann (2011). Our quantitative results demonstrate that PCR inhibition only marginally influenced the real-time detection of *Aphanomyces astaci*. Since the same DNA samples were used for the semi-nested PCR, inhibition is not a likely explanation for the lower sensitivity observed. Primer 42 of the semi-nested PCR (Oidtmann et al. 2006) includes an 'A' insertion at Position 11 that is missing in publicly available sequences of *A. astaci* strains other than M96/1 (AY310499), suggesting a sequencing error or intraspecific variation in Strain M96/1 (see Fig. 4). However, no difference in sensitivity was observed when a primer (42v2) without this mismatch was tested (Tuffs & Oidtmann 2011), suggesting that the mismatch in primer 42 does not negatively influence assay sensitivity. More surprisingly, removing this mismatch had a drastic influence on assay specificity, and was therefore not recommended by Tuffs & Oidtmann (2011). Hence, the observed difference in sensitivity between conventional PCR and real-time PCR observed in the present study and by Tuffs & Oidtmann (2011) is more likely explained by technological and fragment size differences. More DNA is required to visualise a PCR band on a conventional agarose gel compared to detection by real-time PCR where just a few copies generate a signal. Further, the real-time and single-round conventional PCR assays target 57 bp and 569 bp, respectively. The detection ability of the conventional PCR assay is therefore more vulnerable to DNA degradation.

The observation that 16 and 32% of the spiny-cheek crayfish and signal crayfish, respectively, fell into Agent Level A1 may indicate that an even larger proportion of crayfish individuals in the present study were carriers of *Aphanomyces astaci*. Only soft

abdominal cuticle was analysed in the present study, while additional analyses of the tail fan (including telson) or walking leg joints (Oidtmann et al. 2006, Strand et al. 2011) could have increased the observed number of carriers. Vrålstad et al. (2011) demonstrated significantly higher success of detecting *A. astaci* from tail fan tissues than from soft abdominal cuticle in signal crayfish, and recommended the use of tail fan tissue for *A. astaci* prevalence studies in that species.

The positive samples new to the current study occurred predominantly in signal crayfish from the Czech Republic (Table 2), and increased the frequency of *Aphanomyces astaci* positives from 1% (Kozubíková et al. 2009) to 22% of all the tested signal crayfish from this country. Further, at least one *A. astaci*-carrying individual in all investigated Czech signal crayfish populations was uncovered. Similarly, we detected Agent Level A2 in 1 out of 2 investigated signal crayfish individuals from Slovakia. Invasions of American crayfish species were only recently reported from that country (Janský & Kautman 2007, Petrusek & Petrusková 2007), and, although the presence of the crayfish plague pathogen could be suspected, our analysis is the first to support this assumption with molecular data.

Intriguingly, 2 signal crayfish with very low agent levels (A2) were found even in a population where noble crayfish and signal crayfish had coexisted for at least 10 yr without any sign of crayfish plague outbreak (locality Ráček II). We cannot rule out the presence of an avirulent *A. astaci* strain, or, alternatively, a false positive result due to cryptic *Aphanomyces* species diversity or minor laboratory-induced contamination. However, the results could also imply that crayfish plague outbreaks may be delayed for years in localities where European and American crayfish coexist, if the level of *A. astaci* infection in the carrier population is very low. Skov et al. (2011) recently reported that among 60 individuals from a mixed population of signal crayfish and noble crayfish in Denmark, no *A. astaci*-positive individuals were detected with the real-time PCR method of Vrålstad et al. (2009). Skov et al. (2011) acknowledged that it is impossible to declare a signal crayfish population free of infection, but assumed that the investigated signal crayfish population posed a minor, if any, threat for disease transmission. The present study underlines that extreme caution must be exercised before any American crayfish population is reported free of *A. astaci* infection. Sampling effort and diagnostic procedures will influence the probability of detecting *A. astaci* in populations with very low agent prevalence. If future studies confirm that even mixed populations of American and European crayfish may represent minor infection reservoirs of *A. astaci*, it may be only a matter of time before the conditions allow the crayfish plague to strike, leaving the coexisting indigenous European crayfish at constant risk.

Our semi-quantitative data for a large set of American crayfish samples are in concordance with the findings of Vrålstad et al. (2009) where most positive samples of American crayfish showed agent levels between A2 and A3. However, our spiny-cheek crayfish samples sometimes contained higher levels of pathogen DNA (A4 or A5). Such levels correspond to those found in noble crayfish that had suffered mortality from crayfish plague (Vrålstad et al. 2009). We also found a positive correlation between the prevalence of *Aphanomyces astaci*-positive individuals in American crayfish populations and the agent levels for each individual. This is presumably due to increased numbers of *A. astaci* zoospores in environments with higher *A. astaci* prevalence, which, in turn, increases infection probability.

The high sensitivity of the real-time PCR method shows that the previous results of *Aphanomyces astaci* detection based on conventional PCR have suffered from false negatives. However, the extreme sensitivity of real-time PCR is a challenge concerning laboratory contamination and requires excellent laboratory practices. Further, false positives are not revealed unless sequenced, and putative new strains of *A. astaci* may fail to be detected by real-time PCR alone. To avoid these pitfalls, conventional PCR allowing sequencing should be performed in parallel with real-time PCR when appropriate. The present study demonstrates that this combination is beneficial and may uncover erroneous results and increase our understanding of the pathogen distribution patterns.

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Appendix 1. Table A1. Overview of DNA isolates in which *Aphanomyces astaci* was detected above the limit of quantification (LOQ = 50 PCR-forming units [PFU]). Origin indicates crayfish species (Olim: *Orconectes limosus*; Plen: *Pacifastacus leniusculus*) and country (CZ: Czech Republic; H: Hungary). Acceptable quantification (AQ): the difference in cycle threshold values (ΔCt) between the 10-fold diluted and the undiluted DNA replicate is 3.32 (±0.5). Here, the given PFU-value per sample is calculated as the means of the PFU-value of undiluted DNA and the PFU-value of 10-fold diluted DNA multiplied by 10. A ΔCt < 2.82 indicates minor inhibition (MI; data in bold). The PFU-value in these cases is not accurate, but based on the 10-fold diluted PFU estimate (multiplied by 10). A ΔCt > 3.82 indicates that the 10-fold dilution is out of range (OR; data in italics). The PFU-value is uncertain and solely based on the undiluted DNA sample

Origin	Sample	Agent level	ΔCt	PFU	Quantitative evaluation
Olim-CZ	SME12	A5	3.47	45695	AQ
Olim-CZ	SME37	A4	3.68	8297	AQ
Olim-CZ	SME36	A4	3.55	6611	AQ
Olim-CZ	SME17	A4	3.38	4623	AQ
Olim-CZ	KOR1	A4	3.72	2420	AQ
Olim-CZ	SME9	A4	*4.01*	*2406*	*OR*
Olim-CZ	KOR2	A4	3.74	2218	AQ
Olim-CZ	LAB6	A4	3.82	2116	AQ
Olim-CZ	SME21	A4	3.67	2001	AQ
Olim-CZ	SME27	A4	3.23	1853	AQ
Olim-CZ	SME7	A4	3.6	1807	AQ
Olim-CZ	SME11	A4	3.34	1476	AQ
Olim-CZ	SME35	A4	3.72	1432	AQ
Olim-CZ	PRU11	A4	*3.83*	*1330*	*OR*
Olim-CZ	JIC14	A4	3.54	1213	AQ
Olim-CZ	SME15	A4	3.52	1028	AQ
Olim-CZ	SME10	A3	3.38	901	AQ
Olim-CZ	SME8	A3	3.45	846	AQ
Olim-CZ	LAB3	A3	3.42	710	AQ
Olim-CZ	JIC3	A3	3.54	703	AQ
Olim-CZ	PRU6	A3	*4.02*	*629*	*OR*
Olim-CZ	JIC15	A3	*3.93*	*572*	*OR*
Olim-CZ	SME26	A3	3.03	538	AQ
Olim-CZ	PRU7	A3	3.61	504	AQ
Olim-CZ	SME30	A3	3.36	452	AQ
Olim-CZ	SME19	A3	3.21	434	AQ
Olim-CZ	SME34	A3	3.41	406	AQ
Olim-CZ	SME5	A3	3.47	390	AQ
Olim-CZ	JIC4	A3	3.68	365	AQ
Olim-CZ	DEC3	A3	3.31	336	AQ
Olim-CZ	PRU5	A3	3.39	328	AQ
Olim-CZ	SME6	A3	3.67	296	AQ
Olim-CZ	PSO9	A3	**2.78**	**294**	**MI**
Olim-CZ	LAB2	A3	3.64	282	AQ
Olim-CZ	SME4	A3	3.24	281	AQ
Olim-CZ	SME3	A3	3.28	263	AQ
Olim-CZ	FAR2	A3	*5.09*	*253*	*OR*
Olim-CZ	JIC5	A3	*4.05*	*247*	*OR*
Olim-CZ	PRU9	A3	3.7	239	AQ
Olim-CZ	JIC9	A3	*3.84*	*228*	*OR*
Olim-CZ	SME13	A3	3.18	208	AQ
Olim-CZ	SME25	A3	3.56	186	AQ
Olim-CZ	JIC12	A3	3.79	181	AQ
Olim-CZ	SME18	A3	**2.8**	**175**	**MI**
Olim-CZ	JIC11	A3	3.09	162	AQ
Olim-CZ	PRU1	A3	3.38	162	AQ
Olim-CZ	SME29	A3	2.86	160	AQ
Olim-CZ	PRU3	A3	*4.38*	*151*	*OR*
Olim-CZ	PRU10	A3	*4.82*	*137*	*OR*
Olim-CZ	SME16	A3	2.85	135	AQ
Olim-CZ	SME40	A3	3.48	130	AQ
Olim-CZ	JIC8	A3	*4.46*	*129*	*OR*
Olim-CZ	SME20	A3	3.31	123	AQ
Olim-CZ	SME22	A3	3.3	119	AQ
Olim-CZ	JIC13	A3	*4.39*	*108*	*OR*
Olim-CZ	SME2	A3	*4.27*	*108*	*OR*
Olim-CZ	SME33	A3	3.27	99	AQ
Olim-CZ	PSO12	A3	**2.71**	**95**	**MI**
Olim-CZ	PSO18	A3	3.4	93	AQ
Olim-CZ	PSO2	A3	*5.55*	*91*	*OR*
Olim-CZ	PRU8	A3	*5.23*	*86*	*OR*
Olim-CZ	PRU2	A3	3.73	85	AQ
Olim-CZ	SME31	A3	*4.2*	*85*	*OR*
Olim-CZ	PRU4	A3	3.74	83	AQ
Olim-CZ	MAL5	A3	*4.23*	*79*	*OR*
Olim-CZ	JIC10	A3	3.18	78	AQ
Olim-CZ	JIC7	A3	3.71	76	AQ
Olim-CZ	JIC6	A3	3.65	75	AQ
Olim-CZ	PRO08	A3	3.58	73	AQ
Olim-CZ	LAB5	A3	*4.36*	*62*	*OR*
Olim-CZ	PSO5	A3	3.34	61	AQ
Olim-CZ	PSO11	A3	3.57	58	AQ
Olim-CZ	MAL6	A3	*3.96*	*53*	*OR*
Olim-H	G1	A3	*4.19*	*238*	*OR*
Plen-CZ	NAD47	A3	2.89	96	AQ
Plen-CZ	NAD12	A3	*6.45*	*87*	*OR*
Plen-CZ	NAD16	A3	*6.8*	*68*	*OR*
Plen-H	HRI14	A3	3.55	57	AQ
Plen-H	HRI3	A3	*4.32*	*51*	*OR*

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