Identification of exotic North American crayfish in Europe by DNA barcoding

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ABSTRACT

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). 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, Kőszeg	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
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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.

| 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
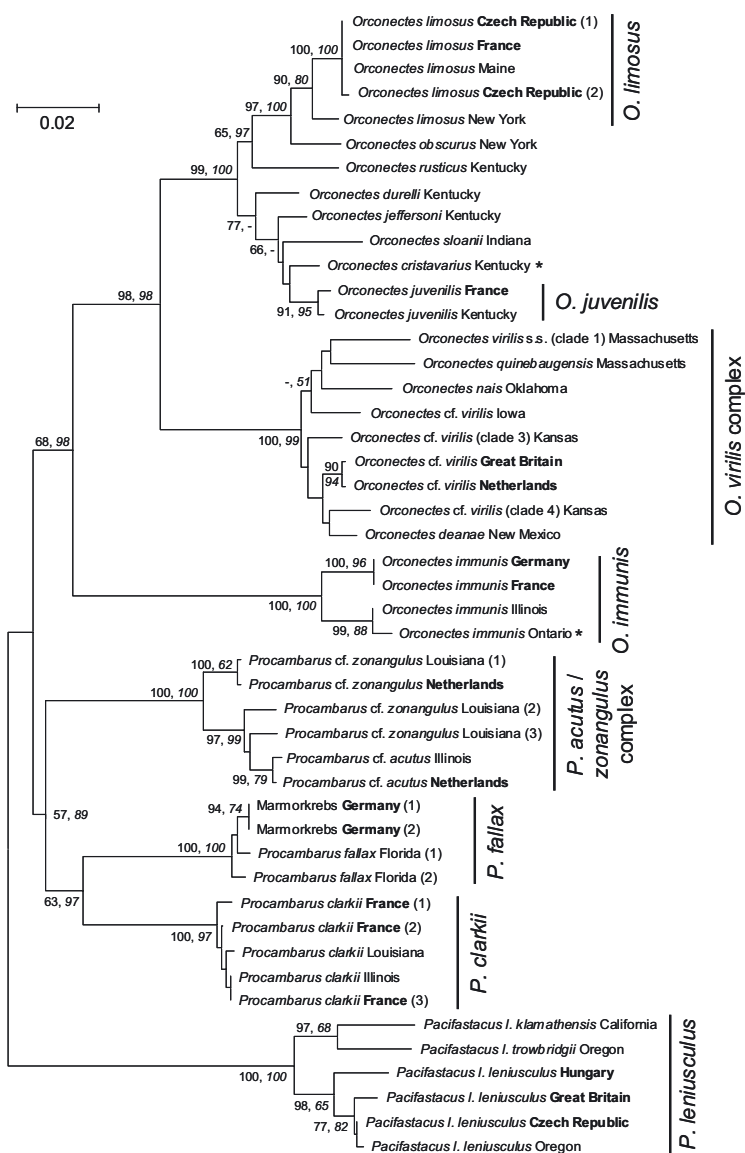
Figure 1

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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