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High regional differentiation in a North American crab species throughout its native range and invaded European waters: a phylogeographic analysis

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Abstract *Rhithropanopeus harrisii* (Gould 1841) has a native distribution from New Brunswick (Canada) to Veracruz (Mexico) and is considered an invasive species in northwestern North American (Oregon and California), South American (Brazil) and European estuaries and rivers. In Europe, it was observed for the first time in 1874, in The Netherlands. We sequenced and analyzed part of the cytochrome oxidase subunit I gene (mitochondrial DNA) of eight populations, three from the east coast of the United States of America (USA) and five from Europe, in order to assess their genetic diversity and to determine a potential founder population. European populations are characterized by a lower number of haplotypes than the whole native region of the eastern USA, suggesting that genetic bottlenecks occurred during the European colonisation. Along the North American East Coast,
there is evidence of clearcut genetic heterogeneity, New Jersey being the most similar population in its genetic structure to the postulated Europe-founding population. Also the different European populations are heterogeneous and there is a tendency of higher genetic diversity in the populations founded earlier. *R. harrisii* is still in the process of expansion in Europe and may have been introduced once or repeatedly by different invasion mechanisms. The pronounced lack of gene flow among populations is of great ecological significance, since it may facilitate rapid adaptation and specialization to local conditions within single estuarine systems.

Keywords *Rhithropanopeus harrisii* · Invasive species · mtDNA diversity · Genetic bottleneck · Population isolation · Larval retention

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Introduction

Exotic species are one important factor in the global diversity crisis, changing community composition and ecosystem functioning. Besides, they often cause significant economic damage (Cohen and Carlton 1998; Stachowicz et al. 1999; Grosholz 2002). For this reason, there is great interest in understanding why and how successful invasions occur (Stachowicz et al. 1999). Some studied examples, mainly for aquatic invertebrates, range from ecological impact assessment (e.g., Marck and d'Antonio 1998;

Grosholz 2002) and invasion dynamics (e.g., Neubert et al. 2000) to invasion pathways and genetic characterization (e.g., Müller 2001; ICES 2005). Molecular tools proved to be very useful in this context, since they allow genetic comparisons among any *taxa* or populations, which is crucial when reconstructing invasion histories (Avise 2004).

Decapod crustaceans are a key group in benthic communities and present characteristics useful for long distance migrations, so it is not surprising that many species have been associated with different invasion processes (Rodríguez and Suárez 2001). *Carcinus maenas*, outside of Europe (see Yamada et al. 2005), and *Eriocheir sinensis*, in Europe (see Hänfling et al. 2002), are good examples of the ecological impact that exotic crab species might have on native communities. The latter species is an ecological and economic threat in northern Europe owing to its burrowing activities in the banks of estuaries and predation on commercial fish (Hänfling et al. 2002). But according to Cabral and Costa (1999), *E. sinensis* in the Tagus Estuary (Portugal) was not a hazard in the 1990s and it was even used as a food source.

Rhithropanopeus harrisii (Gould 1841) is a euryhaline decapod crustacean that presents a larval retention mechanism within estuaries (Cronin 1982; Cronin and Forward 1986). It is easy to collect and, because its larvae show resistance to lower salinities, its ecological and physiological requirements are well studied (e.g., Alvarez et al. 1995; Grosholz and Ruiz 1995; Cripe et al. 2003). The native distribution of this species extends from New Brunswick (Canada) to Veracruz (Mexico). It is considered an invasive species in northwestern North American (Oregon and California), central and southeastern South American (Panama and Brazil), and European estuaries and rivers (Christiansen 1969; Roche and Torchin 2007). In Europe it was observed for the first time in The Netherlands in 1874 (Wolff 1954), and in the following years it was discovered in many other European countries/regions (mentioned chronologically): southeastern France (Adour River); Bulgaria, Romania and Russia (Black Sea area); Germany; Poland; Denmark; north of France (Normandy); Sea of Azov and Caspian Sea (Christiansen 1969); Spain (Cuesta et al. 1991); Portugal (Gonçalves et al. 1995); Italy (Mizzan and Zanella 1996 in Mizzan 1999), and Mediterranean France (Galil et al. 2002). Today it is distributed from the Caspian and Black Sea throughout the Mediterranean and European Atlantic all the way to the Baltic Sea. According to Gonçalves et al. (1995), *R. harrisii* has a patchy distribution along the coast of the Iberian Peninsula, being present only in the Mondego Estuary (central Portugal) and in the Guadalquivir River (south Atlantic coast of Spain). Recent observations of the presence of this species in the Tagus Estuary have broadened its Iberian distribution. It is also known that this species carries a herpes-like virus (Payen and Bonami 1979) and that this kind of virus can be fatal for juvenile oysters, lobster and shrimp populations (Renault 1998; Shields and Behringer 2004). Therefore, it would be of great importance to evaluate the status of this exotic species, in order to monitor its future spread and to understand its genetic and ecological characteristics. This can be achieved through knowledge of the invasion history and population genetic characterization of *R. harrisii* along European coasts.

The goals of this study are thus to verify (1) if the European populations of this species can be attributed to only one colonisation event or were introduced several times independently from different areas of origin and (2) if haplotype frequency differentiation exists between European populations, which would imply lack of gene flow and independent evolution after the respective invasions.

Materials and methods

We had access to eight populations; three from the east coast of the United States of America (USA) and five from Europe (see Table 1, Fig. 1). The sequences from one population of the USA, Neuse River (North Carolina), were obtained directly from GenBank, derived from a study on the genetic structure of the invasive populations in California and Oregon as compared to the Neuse River population (Petersen 2006).

Genomic DNA was extracted from the muscle tissue of walking legs using the Puregene kit (Gentra Systems). Parts of the mitochondrial cytochrome-oxidase subunit I (COI) gene, with a length of 650 bp, were amplified through touch down polymerase chain reaction (PCR; 40 cycles: 45 s at 94°C, denaturating; 1 min at 52–48°C, annealing; 1 min at 72°C, extension). Primers used were: COL14

Table 1 Sampled populations and number of individuals of *Rhithropanopeus harrisii* used for molecular analyses, with numbers of individuals for each haplotype in each sequence analyzed

Populations	No. of individuals	Haplotypes	No. of individuals per haplotype
Europe			
Poland (Pol)	20	A	11
		B	8
		C	1
The Netherlands (Nth)	16	A	5
		C	6
		S	2
		Nth11	1
		Nth12	1
		Nth20	1
Portugal (Por)	20	B	9
		C	10
		Por19	1
Spain (Spn)	20	A	8
		B	2
		C	4
		U	1
		P	3
		R	2
Bulgaria (Bul)	20	A	18
		T	2
USA			
New Jersey (NJ)	19	B	2
		C	9
		U	5
		NJ16	1
		NJ12	1
		NJ13	1
North Carolina: Neuse R. (N)	28	A	1
		All N no. of haplotypes	27
Louisiana (Lou)	20	M	8
		O	5
		Lou3	1
		Lou8	1
		Lou10	1
		Lou11	1
		Lou15	1
		Lou17	1
		Lou18	1
Total	163	51	163

(5′-GCT TGA GCT GGC ATA GTA GG-3′; identical to internal forward primer of Roman and Palumbi 2004) and the new primer COH11R (5′-GCC TTT GGW ATA ATT TCT CA-3′). Sequences were obtained by cycle sequencing, run in an automated sequencer (for details see Reuschel and Schubart
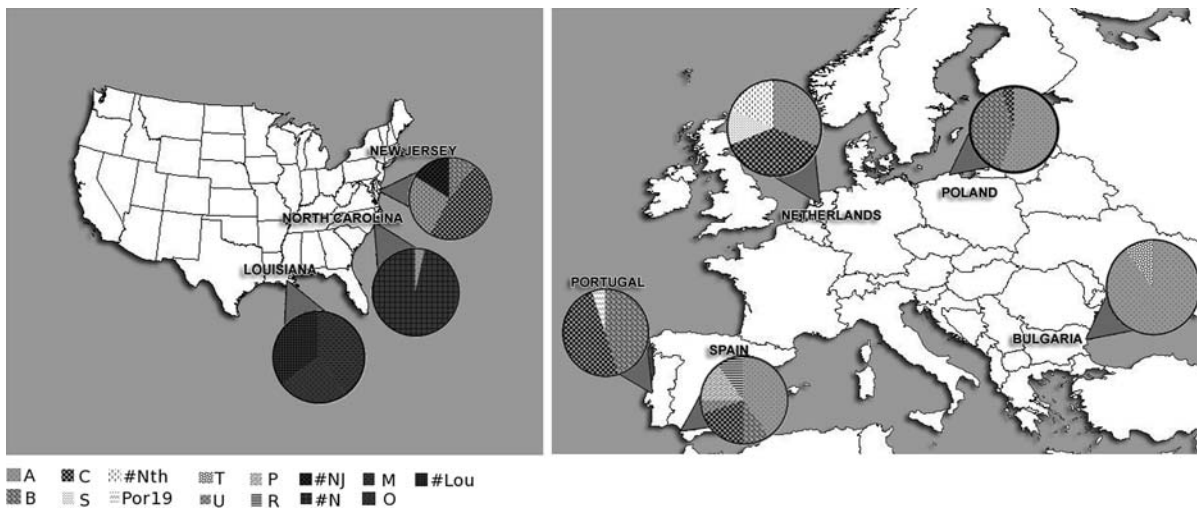
Fig. 1 Origin of analyzed populations of *Rhithropanopeus harrisii* along the east coast of the USA and in Europe; relative distribution of the haplotypes in each population. *Dark grey background*: USA haplotypes, *light grey background*: shared haplotypes and *blank background*: European haplotypes

2006), and read and edited with BioEdit (Hall 1999). The sequences are available through GenBank (accession numbers: FJ517398–FJ517532).

The genetic diversity (nucleotide diversity: π and haplotype diversity: h) was assessed through DnaSP 4.0 (Rozas and Rozas 1999). The following additional tests were run with Arlequin 3.11 (Excoffier et al. 2005): AMOVA analysis (Φ statistics), for the analysis of variance; neutrality tests (Tajima's D and Fu's Fs), that test whether sampled populations follow the neutrality model (i.e., genetic variability maintained by a balance between mutations and genetic drift; Avise 2004); mismatch distribution analyses using the minimization of the sum of squared deviations (SSD) between the observed mismatch distribution and its expectation, under the model of sudden expansion, discriminating distributions that result from expansion or from stationary populations (Harpending et al. 1993; Schneider and Excoffier 1999); and a raggedness index that quantifies the smoothness of the observed mismatch distribution, showing low values when populations underwent an expansion event, and high values when the populations are stationary or suffered a bottleneck (Harpending et al. 1993; Harpending 1994). The model of DNA substitution that best fit our data (providing a gamma value for the Arlequin analyses) was chosen using the software Modeltest 3.6 (Posada and Crandall 1998).

A statistical parsimony network of haplotypes (Templeton et al. 1992) was constructed using the program TCS (Clement et al. 2000).

Results

Obtained sequences were trimmed to a length of 523 bp in order to make them comparable with the ones from GenBank (Neuse River population). In that set of sequences, a total of 163 sequences were analyzed, revealing 51 different haplotypes with 47 variable sites, of which 30 are parsimony-informative; 55 mutations were detected, of which 7 are non-synonymous. The haplotype and nucleotide diversities within each population revealed that the European populations tend to have lower diversities than the American ones, especially than the population from the Neuse River in North Carolina (Table 2). However, the Neuse River population presents exceptionally high values of genetic diversity and a comparison of the European populations with the New Jersey (NJ) population shows that the European haplotype diversity is not very different from the native one. The Netherlands and Spain present even higher values of both haplotype diversity and nucleotide diversity than NJ (Table 2). Usually, the haplotype diversity and nucleotide diversity follow the same pattern in the studied populations. For Poland this was not quite the case, since this population presents a

Table 2 Measures of genetic diversity, neutrality tests and mismatch distribution analyses of the sampled populations of *Rhithropanopeus harrisii*

Populations	N	No. of haplotypes	No. of polymorphic sites	Haplotype diversity (h ± SE)	Nucleotide diversity (π ± SE)	Tajima's D	P	Fu's Fs	P	SSD	P	Raggedness index	P
Europe													
Poland	20	3	5	0.563 ± 0.063	0.0018 ± 0.001	−1.06	0.177	1.033	0.73	0.036	0.15	0.221	0.1
Netherlands	16	6	9	0.783 ± 0.072	0.006 ± 0.001	0.852	0.808	0.714	0.671	0.067	0.1	0.105	0.45
Portugal	20	3	5	0.574 ± 0.055	0.005 ± 0.000	1.808	0.969	3.638	0.957	0.273	0.0	0.675	0.000
Spain	20	6	7	0.795 ± 0.065	0.005 ± 0.001	1.334	0.93	0.667	0.67	0.051	0.25	0.101	0.25
Bulgaria	20	2	1	0.189 ± 0.108	0.000 ± 0.000	−0.592	0.258	−0.097	0.211	0.015	0.4	0.422	0.5
USA													
New Jersey	19	6	5	0.725 ± 0.083	0.003 ± 0.001	−0.059	0.506	−1.349	0.14	0.009	0.4	0.062	0.85
Neuse River (NC)	28	28	29	1 ± 0.000	0.009 ± 0.001	−1.389	0.072	−25.667	<0.001	0.002	0.65	0.022	0.55
Louisiana	20	9	10	0.8 ± 0.073	0.003 ± 0.001	−1.675	0.033	−4.715	<0.001	0.001	0.9	0.06	0.45

Neutrality tests and mismatch analyses present their *P* values

haplotypic diversity similar to Portugal, but its nucleotide diversity is much smaller (Table 2).

The analyses of variance (Table 3) show that the largest fraction of the variation resides among populations within regions (Europe and USA; >53%). Within populations there is also a great amount of variation, but this value is not significant (*P* = 0.43). The contrary is observed among regions, where the percentage of variation is small, though significant (*P* < 0.0001), meaning that populations differ significantly inside each region (Europe and USA), but these regions differ from each other at a small level.

From the neutrality tests we can observe that all populations are close to a mutation-random drift equilibrium (neutral model), since the model was not rejected, except for two populations, Neuse River and Louisiana (Table 2). The first population yields a very large negative value from the Fu's Fs, which means, because the test is sensitive to population size changes (Excoffier et al. 2005; Fu 1997), that probably the Neuse River population has experienced a demographic expansion. From the pairwise mismatch analyses we obtain the same kind of result, since the Neuse River population does not reject the model of sudden expansion (SSD = 0.002; *P* = 0.65). Unfortunately, the raggedness index is not significant, but, its low value (mean value of 0.208) should be noted. Taking into account the mismatch distribution graph for the Neuse River population, a unimodal distribution is clear (Fig. 2), so this population has been growing for a long time or had a sudden expansion event in the past (Harpending 1994; Rogers and Harpending 1992). Also the minimum spanning network, produced by TCS, for the Neuse River population (all haplotypes identified with "N") shows a star-like pattern, which means that this population must have had an expansion event in the past (Slatkin and Hudson 1991; Fig. 3). Some of these results were also obtained by Petersen (2006), with the exception of the neutrality test results and the mismatch distribution graph. The other population that was not in agreement with the neutral model was the Louisiana population. In this case, both tests, Tajima's D and Fu's Fs, give significant *P* values. The later test is not as negatively large as for the Neuse River population, and Tajima's D gave a significant negative value that might be due to a large insertion/deletion polymorphism or to a bottleneck event (Tajima 1989a; Table 2). Similarly to the Neuse River population,

Table 3 Hierarchical analysis of variance of a squared matrix of distances between all *Rhithropanopeus harrisii* haplotypes

Source of variation	Variance components	% Variation	P value	Φ-statistics
Among regions	0.00730	0.31	<0.0001	$\Phi_{SC} = 0.53435$
Among populations within regions	1.27463	53.27	<0.0001	$\Phi_{ST} = 0.53577$
Within populations	1.11075	46.42	0.43011	$\Phi_{CT} = 0.00305$

the model of sudden expansion cannot be rejected for the Louisiana population. Once again, the raggedness index is not significant but shows a low value, and through the mismatch distribution graph we can observe a unimodal distribution (Table 2; Fig. 2). TCS analysis also resulted in a star-shape network of the Louisiana haplotypes, even if they are not as numerous as in the Neuse River population (Fig. 3). All of these tests suggest that a population expansion might have occurred, so the fact that the Tajima's D was significantly negative for Louisiana cannot be attributed to a bottleneck event. As for the other North-American population, New Jersey, we cannot reject any of the neutrality tests, nor the model of sudden expansion. The raggedness index value does not differ significantly from the model, but is also low relative to the mean value, indicating growth for a long time or a burst of expansion (Harpending 1994). As shown by the TCS network, all the haplotypes corresponding to NJ are among other European haplotypes, like B, C, and U (Fig. 3), making it difficult empirically to observe a star-like shape.

For the European populations, it was not possible to reject the neutrality tests and the sudden expansion model. Portugal was the only population that came close to rejecting this model, being at the statistical limit (SSD $P = 0.05$). The raggedness index for Portugal was highly significant, yielding a value that, relative to the mean, can be considered high ($r = 0.675$; $P < 0.001$), so we might infer that it is a stationary or a bottlenecked population that, possibly, did not pass through a sudden population expansion (Harpending 1994). All the other populations did not present significant raggedness indexes.

The TCS network (Fig. 3) shows that the Neuse River (North Carolina) population and the Louisiana animals occupy the opposite extremes of the network and that Louisiana shows four fixed differences with all haplotypes from the American East Coast and Europe.

Three frequent haplotypes, A, B, and C, are predominant. These haplotypes are constituted mainly by European individuals, the exception being individual N8 from Neuse River corresponding to haplotype A and several NJ individuals corresponding to haplotypes B and C (Figs. 1, 3). The U haplotype is represented by Spanish and NJ individuals (Table 1; Fig. 1). In Fig. 1 we can also observe that the haplotypes present in Europe exist also in the NJ population as a great majority, and in the North Carolina population as a much smaller fraction. The Netherlands, Portugal, Spain, and Bulgaria have haplotypes of their own that are not represented in the USA populations (Fig. 1; Table 1).

Discussion

The first goal of this study was to find out if, the European populations of *R. harrisii* can be traced to a single colonisation event or were introduced several times independently from different areas of origin. We show that the NJ population includes individuals with two of the most frequent European haplotypes (B, C). This makes it the most similar population in its genetic structure to the potential founding population of the European colonisation. The fact that the most frequent haplotype (A) from Europe was only found in the USA populations in relatively low frequency (in the Neuse River, North Carolina), can be explained by a sudden spreading of a small founder population in Europe or alternatively by an insufficient sampling of the USA populations. Therefore, we should take these conclusions as preliminary and await a more thorough sampling of native populations. Nevertheless, for the moment, the most likely hypothesis is that the colonisation of European waters was initiated by individuals originating from the northeastern USA, owing to the fact that four distinct haplotypes (A, B, C, and U) are
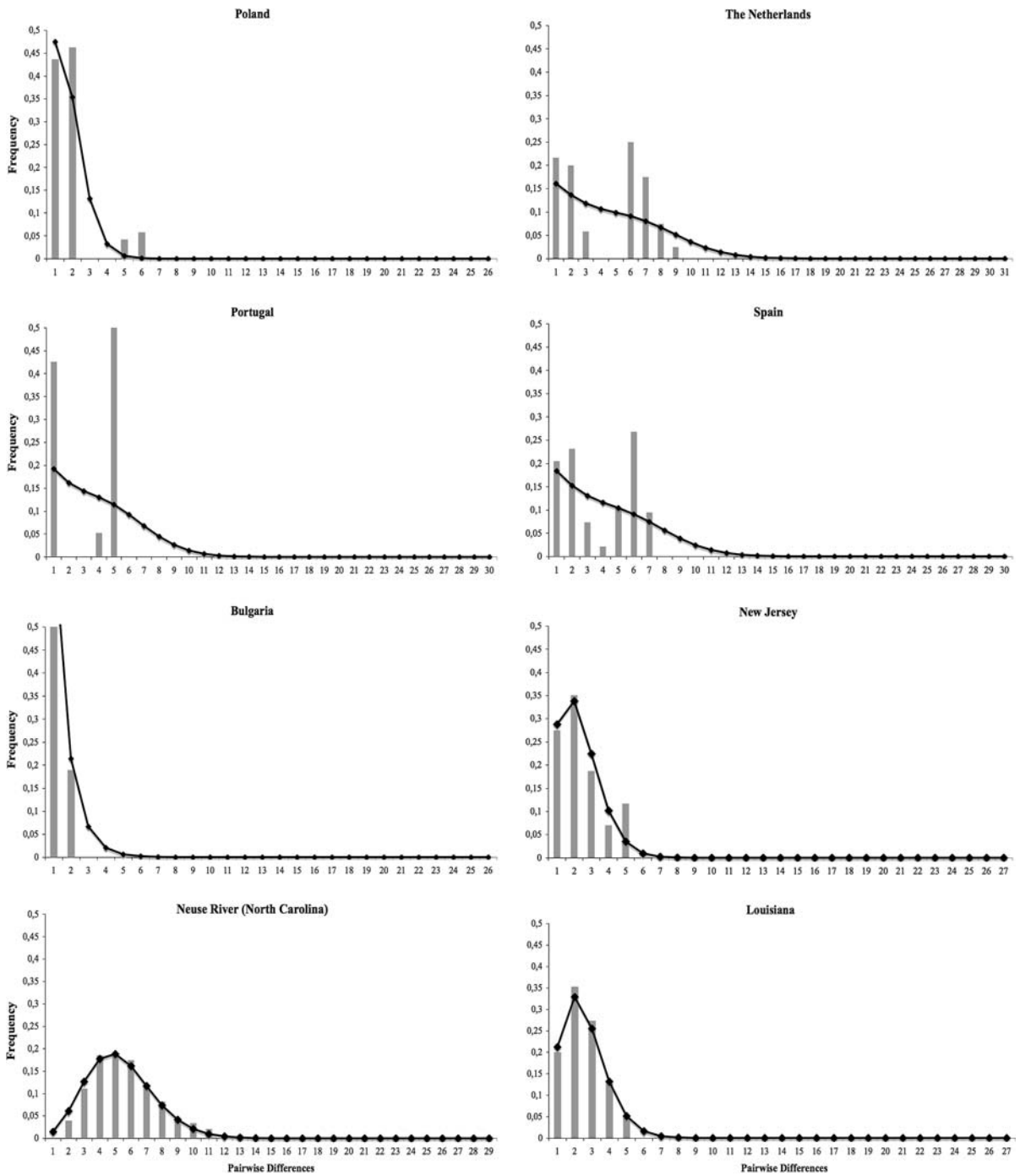
Fig. 2 Pairwise mismatch distributions for each studied population of *Rhithropanopeus harrisii*; expected curve according to a sudden expansion model (*continuous*), observed curve (*bars*)

shared among Europe and the USA populations. This demonstrates that at least four females, not necessarily in the same colonization event, with these different haplotypes, were among the European colonists. In

order to hypothesize that the colonisation could have occurred during a single event, haplotype A, so far only found in Neuse River (North Carolina) among the USA populations, would have to be present also in the NJ
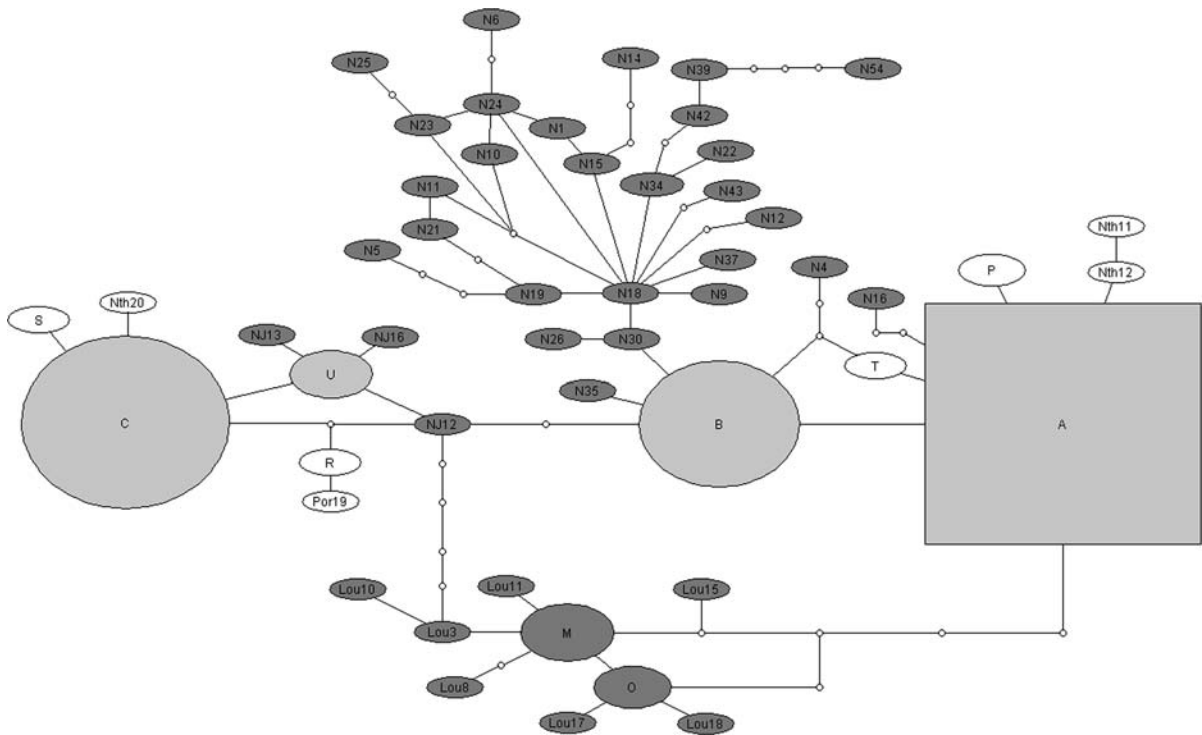
Fig. 3 Minimum spanning network based on a 523 bp fragment of the COI gene of *Rhithropanopeus harrisii* with haplotype A as the proposed ancestral haplotype according to the software TCS. *Dark grey*: USA populations only, *light grey*: haplotypes shared by European and USA populations, *white*: European populations only (see Table 1 for abbreviations and details)

population, or the source population would have to lie between NJ and North Carolina and includes haplotypes A, B, C, and U.

A second goal of this study was to establish whether, differentiation at the level of haplotype frequencies exists between European populations. Our results show that European populations are characterized by a lower number of haplotypes than the assemblage of all native populations in eastern USA. Nevertheless, the Dutch and the Spanish populations showed a similar variability to that of the NJ population and seem to be the most diverse in Europe. This cannot be due to sampling bias, because only 16 sequences from The Netherlands were analyzed. There are two other possibilities: (1) these sampling sites experienced more colonisation events or colonisations by more individuals than, for example, Poland, Portugal, and Bulgaria (which would also explain the similar genetic variability between those European populations and New Jersey); (2) bottlenecks occurred during colonisation from other parts of Europe to Poland, Portugal or Bulgaria. Results for Portugal

showed that this population might not have suffered expansion and given the high raggedness index, and due to the fact that it is not a native region, only a bottleneck event can be assumed. Poland and Bulgaria did not reject the model of sudden expansion, but when bottlenecks last for a long time, their model becomes equivalent to a sudden expansion model, with the same pattern, being unimodal but with curves with steep leading faces (Rogers and Harpending 1992), as can be observed for these two populations (Fig. 2). In Poland and Bulgaria this persistent bottleneck might have acted more strongly on the nucleotide differences (their nucleotide diversity are the lowest in Europe), because probably their population size was very small at the time of colonisation (Tajima 1989b). All this information seems to support the second possibility that bottlenecks occured during European colonisations.

The TCS network and Fig. 1 show also that all European populations (except for Poland) include unique haplotypes, found nowhere else. This could imply that new haplotypes might have evolved in the

newly colonized areas, or a more exhaustive sampling of the USA populations is needed to detect these haplotypes in the founder population. Since COI in decapod crustaceans has an average mutation rate of about 1.4–2.6% per million year (Knowlton et al. 1993; Knowlton and Weigt 1998; Schubart et al. 1998) and *R. harrisii* was first observed in Europe in the nineteenth century (Christiansen 1969), the probability of new mutations is very low, and probably most of these haplotypes also occur in low frequencies in the founder population.

All populations of *R. harrisii* showed a pronounced lack of gene flow (greater fraction of the variability was among populations in each region), and this might be mostly due to the fact that this species shows a larval retention mechanism, retaining the larvae within the vicinity of the parental population (Cronin 1982; Cronin and Forward 1986). This is of great ecological significance, since it may facilitate rapid adaptation and specialization to local conditions within single estuarine systems, which could have contributed to the success of this species outside its native range. For example, in the Portuguese population, which seems to be a stable one, this adaptation could be verified by the authors, since in a period of 10 years this population has moved around 6 km upward in Mondego River, disappearing completely from the previous sampling site.

The invasion pathway for *R. harrisii* to the European coast is assumed to be through ballast water or fouling communities on the hulls of vessels (Christiansen 1969). Ballast water might play an important role for current invasion episodes, but not for the first ones of *R. harrisii*, since it was a mechanism that was not used at the time of the first invasions (Carlton 1985 in Rodríguez and Suárez 2001). Therefore, the first invasions were most likely as a byproduct of live animal shipment for aquaculture like oysters or through fouling communities on ship hulls. Oyster trading seems a viable theory, since it started as early as the seventeenth century between New England and Europe and it was also the invasion vector into California waters (Rodríguez and Suárez 2001; Kirby 2004). The oyster trade to Europe was carried out throughout several northern and southern regions of the USA (Kirby 2004), so it is possible that the founder individuals arrived through several colonisation events, supporting the first hypothesis

mentioned previously, that the Dutch and Spanish populations of *R. harrisii* might have had more founders than other European populations. Transport as fouling material on ship hulls can also be considered a possible invasion mechanism for the first invasions. For example, in German waters about one half of introduced species arrived through the hulls of sea trade ships (Nehring 2005). But since the 1970s, ship hulls and ballast water seem to have similar impacts on invasion processes (Nehring 2001).

An additional new insight from this study is the evidence that the Louisiana population is genetically distinct from the European as well as from the eastern USA populations. This means that this population did not contribute to the European colonisations, and it also constitutes evidence for genetic differentiation along the North American coast. Eventually this difference could be minimized from south to north, if some larvae of *R. harrisii* could be taken by the Gulf Stream current and also from north to south if southward nearshore currents, from Georgia to Cape Canaveral, would transport them. Although more sampling of populations from the northeastern USA would be needed to confirm this dispersal, we can already say that probably these population differences would continue, owing to the larval retention mechanism of this species. Genetic distinctness of Gulf of Mexico populations is well studied in many animal groups along the North American East Coast, most of which have a hybrid/frontier zone in Florida (e.g., Briggs 1974; Reeb and Avise 1990; Young et al. 2002), and this is also known for other crustaceans (e.g., Schubart et al. 2000; Mathews et al. 2002; Young et al. 2002). What leads to this differentiation might depend on several factors, for example, for the *Crassostrea virginica* oyster it depends on the species ecology and their life history, but also on historical and contemporary environmental events (Reeb and Avise 1990).

According to Lee (2002), invasive species present very intriguing characteristics, not only in their dispersal methods, but also in their evolution after the invasion. *R. harrisii* is still a species in expansion and its ecological success might be due to the occupation of vacant niches (Nehring 2000). It is also ominous that *R. harrisii* is a transport vector for a herpes-like virus (Payen and Bonami 1979) that provokes death in oyster, shrimp, lobster, and crab communities (Arzul et al. 2002; Shields and

Behringer 2004). But, for now it remains unknown if *R. harrisii* is a real hazard for European estuarine communities. This is why invasive species are an current and major subject of investigation.

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