



UNIVERSIDADE DE ÉVORA

DEPARTAMENTO DE BIOLOGIA

MESTRADO EM BIOLOGIA E ECOLOGIA DO LITORAL MARINHO

**HIGH REGIONAL DIFFERENTIATION IN A NORTH AMERICAN
CRAB SPECIES THROUGHOUT ITS NATIVE RANGE AND
INVADED EUROPEAN WATERS: A PHYLOGEOGRAPHIC
ANALYSIS WITH REMARKS ON ECOLOGICAL
CONSEQUENCES**

**ACENTUADA DIFERENCIAÇÃO REGIONAL NUMA ESPÉCIE DE CARANGUEJO NORTE
AMERICANA AO LONGO DA SUA DISTRIBUIÇÃO DE ORIGEM E EM ESTUÁRIOS
EUROPEUS: ANÁLISE FILOGEOGRÁFICA E CONSIDERAÇÕES ECOLÓGICAS**

Joana Cristina Projecto Garcia

Master Thesis presented to the University of Évora to obtain the Master degree in “Biologia e
Ecologia do Litoral Marinho”

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ABSTRACT/RESUMO

ABSTRACT

Rhithropanopeus harrisii (Gould 1841) has a native distribution from Canada to Mexico and is considered an invasive species in northwestern American, southeastern American and European estuaries and rivers. In Europe it was observed for the first time before 1874, in The Netherlands.

In this study we analysed a part of cytochrome oxidase subunit I gene of populations from the east coast of United States of America and from Europe, in order to assess to a the genetic diversity of the populations and to determine a potential founder population. Morphometric analyses were also performed using measurements of the carapace.

A genetic bottleneck might have occurred during the European colonization. New Jersey is the most similar population to the potential founding population and there is evidence of genetic heterogeneity along the North American east coast and European coast. It was not possible to discriminate all populations morphometrically. *R. harrisii* is still in expansion and might use several invasion mechanisms.

RESUMO

Rhithropanopeus harrisii (Gould 1841) tem uma distribuição nativa desde o Canadá até ao México e é considerada uma espécie invasora nos rios e estuários da costa noroeste e sudeste do continente americano e na costa Europeia, onde foi descoberto antes de 1874, na Holanda.

Neste estudo uma parte do gene citocromo oxidase subunidade I foi analisada em populações dos EUA e da Europa, de modo a obter a informação sobre a diversidade genética das populações e sobre uma potencial população de origem. Análises morfométricas foram efectuadas através de medições da carapaça.

Um gargalo genético deverá ter ocorrido aquando da colonização Europeia e Nova Jersey é a população que mais se assemelha geneticamente à potencial população de origem. A heterogeneidade genética ao longo da costa este dos EUA e da costa Europeia tornou-se evidente. Pela análise morfométrica não foi possível discriminar as populações. Verifica-se que esta espécie ainda se encontra em expansão e que pode usar vários vectores de invasão.

GENERAL INTRODUCTION

Exotic species are one important factor in the current problems concerning global diversity crisis, changing community composition and ecosystem functioning, many times causing significant economic damage (Cohen & Carlton 1998, Stachowicz et al. 1999, Grosholz 2002). This problem has a huge relevance and involves many biological *taxa*. According to some studies, the community diversity depends heavily on the scale that we are observing, since different factors work at different levels (Levine 2000). So resistance/permission to invasion may depend on several communities' characteristics not always the same in time and space. The study of invasion processes has several advantages like the ability to predict the rates and direction of spread; the evaluation of interventions against invasion spreading and using the invasive species as 'biological tracers' (Johnson & Padilla 1996). For this reason, there is an increasing 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. Mark & D'Antonio 1998, Grosholz 2002), 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 and particularly mtDNA is especially suited to resolve questions at an intraspecific level since it evolves rapidly, revealing recent population history and is a maternally inherited molecule, so that there is no recombination (Avice 2004). The different genes of mtDNA evolve at different rates and it has been shown that cytochrome oxidase, subunit I (COI) can evolve faster than 16S (rRNA) but slower than cytochrome oxidase B (Lynch & Jarrel 1993). By using COI it is possible to assess rapid evolutionary processes and at the same time have deeper phylogenetic insights. Several studies in invertebrates using this molecular marker proved to have reliable results (e.g. Wares and Cunningham 2001, Roman and Palumbi 2004, Vandewoestijne et al. 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 many invasion processes (Rodríguez & Suárez 2001). *Carcinus maenas*, outside Europe (see Yamada 2005), and *Eriocheir sinensis*, in Europe (see Hänfling et al. 2002), represent good examples of the ecological impact that exotic species might infer to native communities. This last species represents an ecological and

economic threat in northern Europe, through its burrowing activities in estuary margins and predation of commercial fish (Hänfling et al. 2002). In other geographical area (e.g. Tagus estuary), this species does not represent a hazard for the moment (Cabral & Costa (1999).

Rhithropanopeus harrisi (Gould 1841), is a euryhaline decapod crustacean that presents a larval retention mechanism that allows the larvae to develop in the surroundings of the parental population (Cronin 1982), which usually thrives in the upper parts of the estuaries in muddy/biogenic bottoms. It is easy to collect and because its larvae present good resistance to lower salinities, its ecological and physiological requirements are well studied (e.g. Alvarez et al. 1995, Grosholz & 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 American (Oregon and California), southeastern American (Brazil) and European estuaries and rivers (Christiansen 1969). In Europe it was observed for the first time in The Netherlands, before 1874 and in the following years it was discovered in most of the European countries: Poland, Germany, Denmark, France, Bulgaria, (Christiansen 1969, Rodríguez and Suárez 2001), Spain (Cuesta et al. 1991), Portugal (Gonçalves *et al.* 1995) and Italy (Mizzan & Zanella 1996 cited in Mizzan 1999). Today it is distributed from the Caspian and Black Sea throughout the Mediterranean and European Atlantic all the way into the Baltic Sea. According to Gonçalves *et al.* (1995a), *R. harrisi* has a patchy distribution along the Iberian Peninsular coast, 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 widened its Iberian distribution (Cabral, pers. comm.).

Invasion processes can transport pathogens and diseases through ballast water (Grosholz 2002), and also invasive species itself can act as vectors of transport and infection to native species. *Rhithropanopeus harrisi* is known that to be a carrier of a herpes-like virus (Payen & Bonami 1979 cited in Shields & Behringer 2004) and this kind of virus can be fatal for lobsters and shrimps populations (Shields & Behringer 2004). Therefore it would be of great importance to evaluate the status of this exotic species, in order to monitor future spreading, molecular and ecological characteristics. This can be achieved through the knowledge of the invasion history and genetic characterization of *R. harrisi* along European coasts. Thus the goals of this study are to evaluate: (1) if the European populations of this species can be attributed to only one

colonization event or were introduced several times independently from different areas of origin; (2) if haplotype frequencies differentiation exists between European populations; and (3) if morphometric analysis corroborate the molecular data.

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ABSTRACT

Rhithropanopeus harrisi (Gould 1841) has a native distribution from New Brunswick (Canada) to Veracruz (Mexico) and it is considered an invasive species in northwestern American (Oregon and California), southeastern American (Brazil) and European estuaries and rivers. In Europe, it was observed for the first time before 1874, in The Netherlands.

In this study, we sequenced and analysed part of the cytochrome oxidase subunit I (COI) 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 to their genetic diversity and to determine a potential founder population. Morphometric analyses were performed using four morphometric measurements: carapace length, carapace width, body height and frontal width.

The molecular analyses showed that European populations are characterized by a lower number of haplotypes than the native populations in eastern USA; and that a genetic bottleneck might have occurred during the European colonization. New Jersey is the most similar population in its genetic structure to the postulated founding population, and along the North American east coast there is evidence of clearcut genetic heterogeneity. Also European populations are heterogeneous and there is a tendency of higher genetic diversity in the first founded populations. It was not possible to consistently separate populations morphometrically. This species is still in the

process of expansion and may be introduced by different invasion mechanisms. The pronounced lack of gene flow among populations is of great ecological significance. 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.

KEY WORDS: *Rhithropanopeus harrisi* · Invasive species · mtDNA · genetic diversity · morphometric

INTRODUCTION

Exotic species are one important factor among the current problems concerning global diversity crisis, changing community composition and ecosystem functioning. Besides, they often cause significant economic damage (Cohen & Carlton 1998, Stachowicz et al. 1999, Grosholz 2002). For this reason, there is a 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. Mack & d'Antonio 1998, Grosholz 2002), 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 (Avice 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 & Suárez 2001). *Carcinus maenas*, outside of Europe (see Yamada 2005), and *Eriocheir sinensis*, in Europe (see Hänfling et al. 2002), represent good examples of the ecological impact that exotic species might infer to native communities. This last species represents an ecological and economic threat in northern Europe due to its burrowing activities in estuary margins and predation of commercial fish (Hänfling et al. 2002). But according to Cabral & Costa (1999), *E. sinensis* in the Tagus Estuary (Portugal) does not represent a hazard for the moment and it is even used as a resource.

Rhithropanopeus harrisi (Gould 1841), is a euryhaline decapod

crustacean that presents a larval retention mechanism within estuaries (Cronin 1982, Cronin & Forward 1986). It is easy to collect and because its larvae present good resistance to lower salinities, its ecological and physiological requirements are well studied (e.g. Alvarez et al. 1995, Grosholz & 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 American (Oregon and California), southeastern American (Brazil) and European estuaries and rivers (Christiansen 1969). In Europe it was observed for the first time in The Netherlands, before 1874 (Rodríguez & Suárez 2001) and in the following years it was discovered in many other European countries: Poland, Germany, Denmark, France, Bulgaria, (Christiansen 1969), Spain (Cuesta et al. 1991), Portugal (Gonçalves et al. 1995) and Italy (Mizzan & Zanella 1996 cited in Mizzan 1999). Today it is distributed from the Caspian and Black Sea throughout the Mediterranean and European Atlantic all the way into the Baltic Sea. According to Gonçalves et al. (1995a), *R. harrisi* has a patchy distribution along the Iberian Peninsula

coast, 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 widened its Iberian distribution. It is also known that this species is a carrier of a herpes-like virus (Payen & Bonami 1979 cited in Shields & Behringer 2004) and that kind of virus can be fatal for lobster and shrimp populations (Shields & Behringer 2004). Therefore it would be of great importance to evaluate the status of this exotic species, in order to monitor future spreading, molecular and ecological characteristics. This can be achieved through the knowledge of the invasion history and population genetic characterization of *R. harrisi* 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 colonization event or were introduced several times independently from different areas of origin; (2) if haplotype frequencies differentiation exists between European populations; and (3) if morphometric differences can be found in the sampled populations.

MATERIAL AND METHODS

Seven populations were sampled; two from the east coast of the United States of America (USA) and five from Europe (see Table 2 and Fig. 1). All the molecular data from the Neuse River (North Carolina, USA) population were obtained directly from GenBank and are derived from a study on the genetic structure of the invasive populations in California and Oregon (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 (SHORT) or 1200 (LONG) base pairs (bp), were amplified through touch down polymerase chain reaction (PCR) (40 cycles:45 sec at 94°C - denaturing, 1min at 52°C to 48°C - annealing, 1min at 72°C - extension). Used primers were: COL14 (5'-GCT TGA GCT GGC ATA GTA GG-3') (identical to internal forward primer of Roman & Palumbi, 2004) and the new primers COL8 (5'-GAY CAA ATA CCT TTA TTT GT-3'), COH11R (5'-GCC TTT GGW ATA ATT TCT CA-3') and COH12 (5'-GGY ATA CCR TTT ART CCT AAR AA-3'). Sequences were obtained by automated sequencing (for details see Reuschel & Schubart 2006) and read and edited

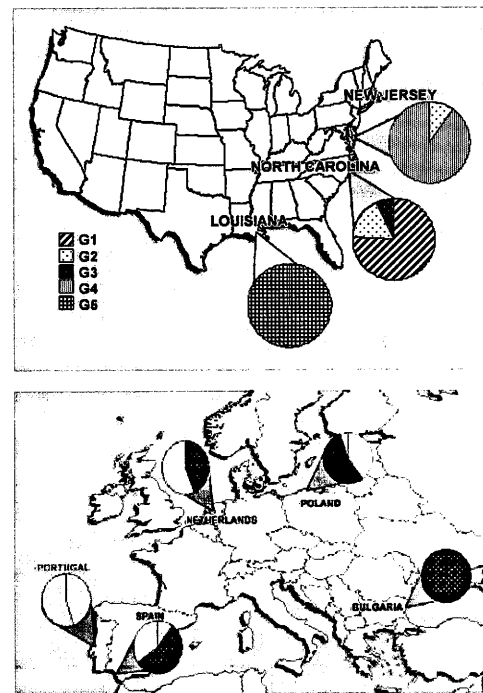


Fig. 1. Sampling sites of *Rhithropanopeus harrisii* along the USA east coast (above) and in Europe (bottom); relative distribution of five the groups with similar haplotypes

with BioEdit (Hall et al. 1999). The genetic diversity within populations (polymorphic sites and haplotype diversity - Hd) was assessed through DnaSP 4.0 (Rozas & Rozas 1999) and through the Φ statistics from an AMOVA analysis (Arlequin 3.0; Excoffier et al. 2006) among populations. A minimum parsimonious haplotype network was constructed using the program TCS (Clement et al. 2000).

A total of 145 individuals (Table 2) were included in a morphometric analysis, in which four measurements

were recorded for each individual. These measurements were carapace length (CL), carapace width (CW), body height (BH) and frontal width (FW). After measuring all the individuals, it became necessary to create size classes because of changing morphometric ratios with size (allometry). The size class “small” (S) included all individuals smaller than the smallest ovigerous female (5mm CW). The other two size classes were “medium” (M), from 5 to 15mm and “large” (L), above 15mm. The univariate analysis, 1-Factor ANOVA (significance level $p=0.017$) and a Discriminant Analysis (multivariate approach) were performed using the ratios of the variables (measurements). The ratios used were: CL/CW, BH/CW and FW/CW. To achieve the assumptions for the statistical analyses the data had to be transformed (arcsin). All analyses were performed using STATISTICA 7.

RESULTS

Molecular Analysis

Two sets of different length sequences were obtained. The first one (SHORT) had the length of 523 bp in order to make our sequences comparable with the ones from GenBank. The second set (LONG) included sequences of 1124 bp. In the SHORT set, a total of 163 plus five single sequences from other populations (RhDe - Delaware, RhNC - North Carolina (Wilmington), RhFL - Florida, RhTX - Texas and RhIT - Italy) were analysed, revealing 54 different haplotypes, 32 parsimony-informative sites and 10 non-synonymous mutations. The haplotype diversity within each population revealed that the European populations have lower diversity than the USA populations, especially than the population from Neuse River in North Carolina (Table 1).

Table 1. Measures of genetic diversity of the sampled populations of *Rhithropanopeus harrisi*

Populations		# of sequences	# of polymorphic sites	# of haplotypes	Haplotypic diversity (Hd)
Europe	Poland	20	5	3	0.563
	The Netherlands	16	9	6	0.783
	Portugal	20	5	3	0.573
	Spain	20	7	6	0.794
	Bulgaria	20	1	2	0.189
USA	New Jersey	19	5	6	0.725
	North Carolina	28	29	28	1.000
	Louisiana	20	10	9	0.800

Table 2. Sampled populations and number of individuals of *Rhithropanopeus harrisi* used for molecular and morphometric analyses; with numbers of individuals for each haplotype

Populations	SHORT				LONG				Morphometric analysis	
	Haplotypes		# haplotypes	#	Haplotypes		# haplotypes	#	#	#
	#	Haplotypes			#	Haplotypes				
Europe	20	A	11	6	Y	4	25			
		B	8		Pol2	1				
		C	1		Z	1				
	16	A	5	3	Nth2	1	17			
		C	6		Nth5	1				
		S	2		Nth6	1				
		Nth11	1							
		Nth12	1							
		Nth20	1							
	20	B	9	5	X	3	20			
USA	20	C	10	1	Z	1				
		Port19	1		Por21	1	23			
	20	A	8	1	Spm1	1				
		B	2							
		C	4							
		U	1							
		P	3							
		R	2							
	20	A	18	11	Y	7	26			
		T	2		W	2				
USA	19	B	2	1	Bul19	1				
		C	9		Bul20	1	19			
		U	4		NI9	1				
		O	2							
		NI12	1							
		NI13	1							
		A	1							
	28	M	27	8						
		O	5							
	20	Lou3	1	2	Lou1	1	15			
	Lou8	1		Lou8	1					
	Lou10	1								
	Lou11	1								
	Lou15	1								
	Lou17	1								
	Lou18	1								

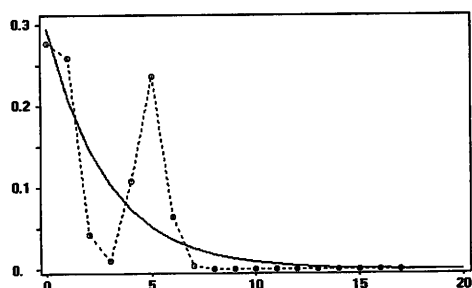


Fig. 2. Pairwise differences between haplotypes; expected curve (continuous) for a natural population, observed curve for *Rhithropanopeus harrisii* (dashed)

Within Europe, Spain and The Netherlands are the populations that present highest Hd values (Table 1).

According to the mean value of overall Φ_{ST} of 0.31 ($p < 0.01$), it is perceptible that there is considerable differentiation among populations, indicating restricted gene flow. Regarding the pairwise Φ_{ST} values (Table 3), the highest ones referred to the Bulgaria population against almost all others. The North Carolina (Neuse River) population presented the lowest values. For the rest of the populations

the values were within the same range (0.20 to 0.35), regardless if they were European or USA populations. Portugal and Poland showed a considerable pairwise Φ_{ST} value, which corroborates restricted gene flow. The lowest value among the European populations was between Spain and The Netherlands. The pairwise mismatch distribution within European populations was also analysed and the observed line for these data does not follow the expected curve for a natural population and presents an abrupt peak in the beginning of the curve, which means that a bottleneck might have occurred (Fig. 2).

The TCS network (Fig. 3) shows that the North Carolina population and the Louisiana group occupy the opposite extremes of the network and that New Jersey presents four consistent differences with Louisiana. This gives evidence that along the USA East Coast there is no genetic homogeneity. Three

Table 3. Pairwise Φ_{ST} values between sampled populations of *Rhithropanopeus harrisii* (all $p < 0.001$). Abbreviation according to Table 1

	POL	NTH	POR	SPN	BUL	NJ	NC	LOU
POL	0							
NTH	0.3320	0						
POR	0.4315	0.3264	0					
SPN	0.3210	0.2107	0.3157	0				
BUL	0.6236	0.5340	0.6184	0.5078	0			
NJ	0.3567	0.2466	0.3514	0.2397	0.5469	0		
NC	0.2080	0.1024	0.2031	0.0995	0.3751	0.1317	0	
LOU	0.3184	0.2080	0.3131	0.2026	0.5052	0.2371	0.0970	0

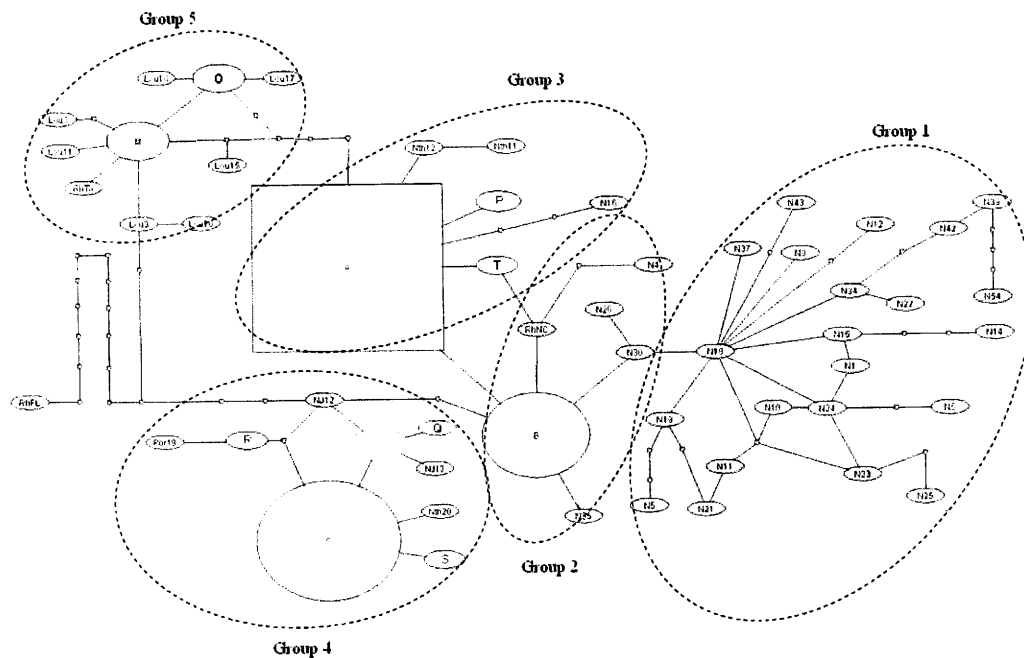


Fig. 3. Minimum parsimonious spanning network based on a 523 bp fragment of the COI gene of *Rhithropanopeus harrisi* with haplotype A as the proposed ancestral haplotype according to the software TCS. Dark grey: USA populations only, light grey: haplotypes shared by European and USA populations, white: European populations only (see Table 1 for abbreviations and details). A, B and C represent frequent haplotypes and all haplotypes with more than one sequence were named with only one letter. Five groups joining similar haplotypes are defined and depicted. Small circles on lines represent missing haplotypes (i.e. additional substitutions)

frequent haplotypes A, B and C are predominant.

These haplotypes are constituted mainly by European individuals, the exception being individual N8 from North Carolina (Neuse River) corresponding to haplotype A and several New Jersey individuals corresponding to haplotypes B and C. The U haplotype is represented by Spanish and New Jersey individuals (Table 2). The only sequence from

Florida presents an elevated number of differences. Fig. 1 represents the geographic origin of groups of similar haplotypes (for definition see Fig 3). It becomes clearly visible that all the groups that were recorded in New Jersey exist in Europe (100%). In contrast only ~24% from the groups present in the North Carolina population is represented in European ones. The most frequent group of haplotypes (G3) in Europe (except for Portugal) is scarce

along USA East Coast, occurring only in the North Carolina population, with a low percentage (~7%), but with 100% frequency in Bulgaria. Again along the USA East Coast genetic heterogeneity is visible.

For the LONG set of sequences a total of 29 plus three more sequences were analysed (RhDe - Delaware, RhNC - North Carolina (Wilmington) and RhIT - Italy) and a haplotype network was constructed (Fig. 4). From this analysis one can observe that the general picture does not change, despite an increase network resolution. Individuals that belonged to the same

haplotypes in the SHORT network, now are separated but still with minor differences between them.

Morphometric Analysis

According to the size class criterion, the distribution of size classes in each population could be compared (Fig. 5). A Discriminant Analysis (Fig. 6) resulted in a separation of size classes (Wilk's Lambda: 0.44; $F(6,28)=23.88$; $p<0.001$; 82% correct classification). To know which ratio is contributing most to the separation of size classes and thus to allometric growth each ratio was plotted against CW, as a categorical variable. All plots clearly showed a

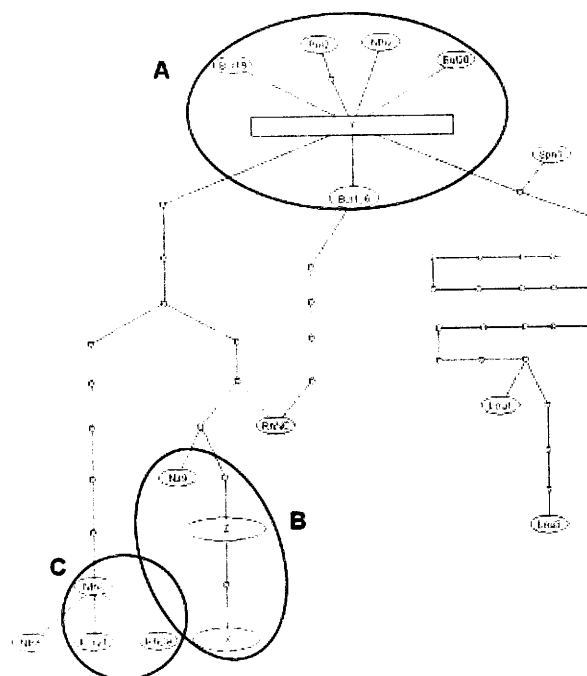


Fig. 4. Minimum parsimonious spanning network based on an 1124 bp fragment of the COI gene of *Rhithropanopeus harrisi*. Dark grey: USA populations, white: European populations, circles and letters A-C represent haplotypes shown in the SHORT network.

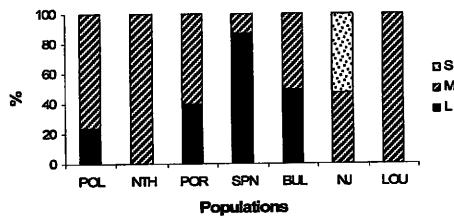


Fig. 5. Size classes frequencies in two native and five European populations of *Rhithropanopeus harrisii*. Abbreviations as in Table 1

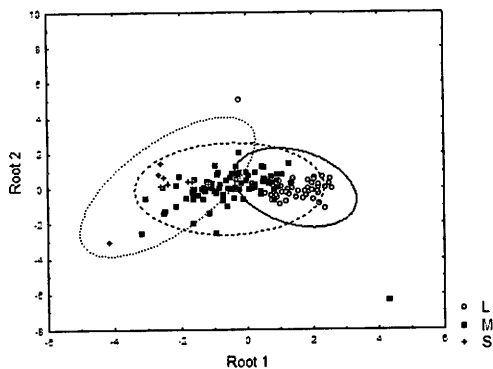


Fig. 6. Ordination diagram of the first two axis of the Discriminant Analysis separating size classes of *Rhithropanopeus harrisii* and showing effects of allometry; each group is highlighted by ellipses (see Material and Methods for abbreviations)

negative correlation between ratios and the CW (Fig. 7) and all associations were significant ($p < 0.001$).

After this analysis it was possible to proceed keeping in mind that our data include allometry effects and that creating size classes would decrease these effects. From the 1-Factor ANOVA analysis of the ratios it was obvious that all were important to separate size classes (Table 4), especially CL/CW and FW/CW (Tuckey test, $p < 0.001$).

The analysis regarding geographical differentiation was carried out only considering the medium size class, which comprised most individuals. A Discriminant Analysis (Fig. 8) was performed and a clear distinction between populations was not perceptible (Wilk's Lambda: 0.4197; $F(18.223) = 4.4697$; $p < 0.001$; 34% of correct classification) despite overall significant differences. Also according

Table 4. 1-Factor ANOVA results from the size classes and population analysis in *Rhithropanopeus harrisii* (for post-hoc results see Results)

Factor	df	F	p
Size Classes			
CL/CW	2	35.56	<0.001
BH/CW	2	10.11	<0.001
FW/CW	2	40.34	<0.001
Populations			
CL/CW	6	2.90	<0.001
BH/CW	6	3.40	<0.005
FW/CW	6	8.97	<0.001

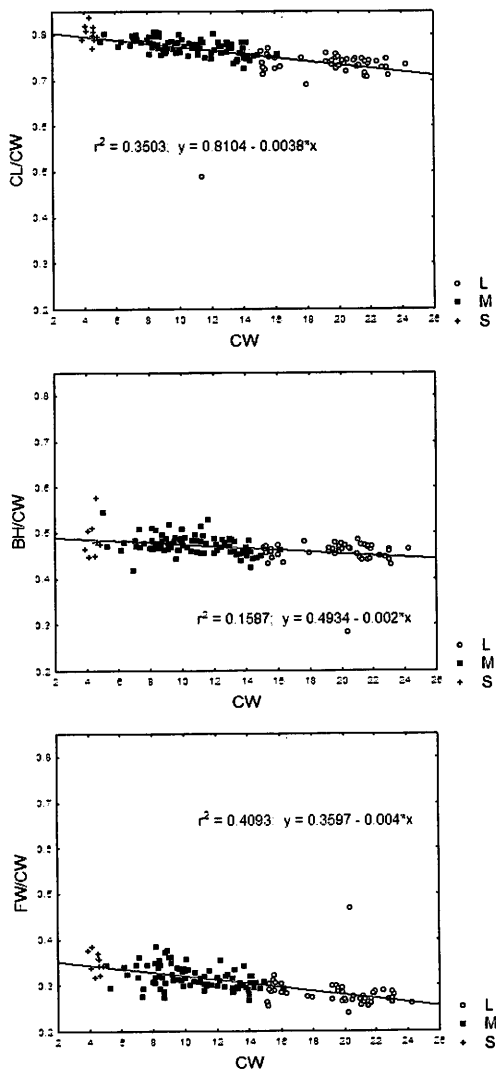


Fig. 7. Regression analysis with regression lines and equations of three morphometric ratios relative to carapace width (CW) (see Material and Methods for other abbreviations) in *Rhithropanopeus harrisii*

to the analysis of variance, all the ratios showed significant differences in the sampled populations (Table 4). The ratios CL/CW and BH/CW were important to differentiate Portugal against New Jersey and The Netherlands, respectively (Tuckey test, $p < 0.001$). The ratio FW/CW showed

differences between Louisiana and all the other populations, except Spain (Tuckey test, $p < 0.001$).

DISCUSSION

The analyses with DnaSP and TCS of 168 COI sequences of 523 bp length showed that European populations are characterized by a lower number of haplotypes, than the native populations in eastern USA. This implies that a genetic bottleneck occurred during the European colonization, which is typical for a long distance colonization event (Sakai et al. 2001).

However, the bottleneck effect might not have been very strong since several haplotypes from USA (three from New Jersey and one from Neuse River populations) could also be found

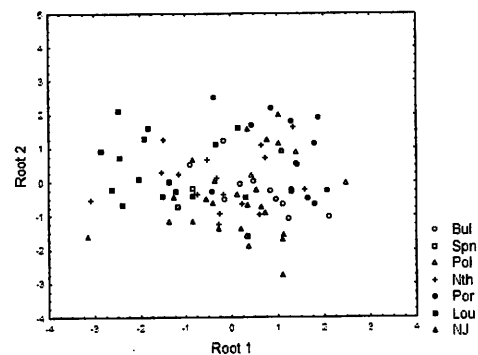


Fig. 8. Ordination diagram of the first two axis of the Discriminant Analysis separating populations of *Rhithropanopeus harrisii* and only considering individuals corresponding to the medium size class (see Table 1 for abbreviations)

in Europe, despite limited sampling of the USA populations. The fact that the most frequent haplotype (A) from Europe only was found in the USA populations in a relatively low frequency (in North Carolina) might be explained by insufficient sampling of the USA populations or to the sudden spreading of a founder population. According to the network, New Jersey is the most similar population in its genetic structure to the potential founding population of the European colonization, since it has representatives with three of the most frequent European haplotypes (B, C and U). Probably the colonization of European waters was initiated by individuals originating from southern New England and the fact that four clearly distinct haplotypes (A, B, C and U) have a high frequency in Europe demonstrates that at least four females with different haplotypes were among the European colonizers.

The Louisiana population is genetically distinct from the European ones, and also from the other USA populations. This means that this population did not contribute to the European colonisations and is presenting important differences from the other USA areas, thereby giving evidence for genetic differentiation

along the North American coast. Genetic distinctness of Gulf of Mexico populations is well studied in many animal groups along the North American east coast, most of them presenting a hybrid/frontier zone in Florida (Briggs 1974, Young et al. 2002) and it is also known for other crustaceans (e.g. Schubart et al. 2000, Mathews et al. 2002, Young et al. 2002).

The TCS network shows that all European populations (except for Poland) include unique haplotypes that are found nowhere else. This could imply that new haplotypes might have evolved in the newly colonized areas, unless a more exhaustive sampling of the USA populations does not provides evidence that these haplotypes are also present in that area. Since COI in decapod crustaceans has an average mutation rate of about 2-2.6% per million year (Knowlton et al. 1993, Schubart et al. 1998) and *R. harrisii* was first observed in Europe in the 19th century (Christiansen 1969), there is not enough time to accumulate many genetic differences. The Bulgarian population has the lowest haplotype diversity, despite of being one of the best sampled populations.

Dutch, Portuguese and Spanish populations seem to be most diverse



within Europe. This cannot be attributed to the fact that they are the best sampled populations, because only 16 sequences were analysed from The Netherlands. There are two other possibilities: 1) these sampling sites suffered more colonization events than, for example, Poland and Bulgaria; 2) additional bottlenecks occurred during colonization from other parts of Europe to areas as Poland or Bulgaria. The fact that these two populations were colonized several years after the first invasion processes, favours the second hypothesis.

The invasion pathway for *Rhithropanopeus harrisi* to reach the European coast is assumed to be through ballast water or with 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. harrisi*, 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 invasion processes were most likely due to live animal shipment for aquaculture, like oysters or through fouling communities on ships' hulls. Historically oyster trading seems a viable theory since it started as early as the 17th century between New England

area and Europe and it was also the invasion vector for the California waters (Rodríguez and Suárez 2001, Kirby 2004). The transport as fouling material on ship hulls can also be considered a possible invasion mechanism for first invasions. For example in German waters about one half of the introduced species arrived through the hulls of sea trade ships (Nehring 2005). But since 1970 ship hulls and ballast water seem to have similar importance for invasion processes (Nehring 2001)

The morphometric results were weak and not always concordant with molecular data. It is very unlikely that, in the short time frame since colonization, evolution of new phenotypes occurred, so that phenotypic plasticity within the genetic framework of this species is the best explanation for the observed variance. Nevertheless, the morphometric analysis was important to characterize this species in the sampled populations and to create awareness that probably there are differences among populations in size classes frequencies and shape parameters. Also Christiansen (1969) pointed out this fact referring to the Dutch animals as larger than the rest of the Europeans representatives. In our study, it was the Spanish population that presented the bigger sizes. Buitendijk

and Holthuis (1949, cited in Christiansen 1969) identified *Pilumnus tridentatus* has *Rhithropanopeus harrisi tridentatus*, maintaining the subspecific name based on differences in morphometry between European and USA forms. Christiansen (1969) rejected this taxonomy and we provide genetic evidence for the uselessness of such a separation. Nevertheless, this analysis shows how important it is to understand regional morphometric differences and the ecological agents that may cause them.

According to Lee (2002) invasive species present very intriguing characteristics, not only in their dispersal methods, but also in the mode they evolve after the invasion process. *Rhithropanopeus harrisi* is still a species in expansion, at least in northern Europe, and its ecological success might be due to the occupation of vacant niches (Nehring 2000). Matters of concern are also the facts that *R. harrisi* is a vector of transport for a herpes-like virus (Payen and Bonami 1979 in Shields and Behringer 2004) that provokes death in shrimp and lobster communities (Shields and Behringer 2004). Until now it is not known if *R. harrisi* represents a hazard in European waters.

The pronounced lack of gene flow among populations might be due mostly to the fact that *Rhithropanopeus harrisi* presents a larval retention mechanism which places the larvae within the vicinity of the parental population (Cronin 1982, Cronin & Forward 1986) diminishing the probability of being taken by ships ballast tanks or of any adult to cling to ship hulls. This probability might increase in storm/heavy flow conditions or probably in the beginning of any colonisation, when the populations are located in the mouth of the estuaries. With time the adult population rises up in the estuary and if the larval retention mechanism is effective the probability of individuals from this population to become invaders is even smaller. If a harbour is located upper in the estuary most of these considerations become invalid.

On a general view these facts are 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. This is why invasive species are an actual and major subject to investigate.

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GENERAL CONCLUSIONS

European populations of *Rhithropanopeus harrisi* were characterized by a lower number of haplotypes, than the native populations in eastern USA. This implies that a genetic bottleneck occurred during the European colonization, which is typical for a long distance colonization event (Sakai et al. 2001). The fact that the most frequent haplotype (A) from Europe only was found in the USA populations in a relatively low frequency (in North Carolina) might be explained by insufficient sampling of the USA populations or to the sudden spreading of a founder population. New Jersey is the most similar population in its genetic structure to the postulated founding population of the European colonization so probably the colonization was initiated by individuals originating from southern New England. The fact that four clearly distinct haplotypes (A, B, C and U) have a high frequency in Europe shows that at least four females with different haplotypes were among the European colonizers.

The Louisiana population did not contribute to the European colonisations since is genetically distinct from the European ones, and also from the other USA populations. This means that this population is presenting important differences from the other USA areas, thereby giving evidence for genetic differentiation along the North American coast. Genetic distinctness of Gulf of Mexico populations is well studied in many animal groups along the North American east coast, most of them presenting a hybrid/frontier zone in Florida (Briggs 1974, Young et al. 2002) and it is also a known fact for other crustaceans (e.g. Schubart et al. 2000, Mathews et al. 2002, Young et al. 2002).

The TCS network shows that several unique haplotypes are only present in European populations. This could imply that new haplotypes might have evolved in the newly colonized areas, unless a more exhaustive sampling of the USA populations does not provide evidence that these haplotypes are also present in that area. Since COI in decapod crustaceans has an average mutation rate of about 2-2.6% per million year (Knowlton et al. 1993, Schubart et al. 1998) and *Rhithropanopeus harrisi* was first observed in Europe in the 19th century (Christiansen 1969), there is not enough time to accumulate many genetic differences. So there are two other possibilities: 1) genetically diverse populations might have suffered more colonization events than populations that are genetically homogeneous; 2) additional bottlenecks occurred during colonization from diverse populations to areas that at present time do not show genetic diversity.

The invasion pathway for *Rhithropanopeus harrisi* to reach the European coast is assumed to be through ballast water or with 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. harrisi*, 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 invasion processes were most likely due to live animal shipment for aquaculture, like oysters or through fouling communities on ships' hulls. Historically oyster trading seems a viable theory since it started as early as the 17th century between New England area and Europe and it was also the invasion vector for the California waters (Rodríguez and Suárez 2001, Kirby 2004). The transport as fouling material in the ship hulls can also be considered a probable invasion mechanism for first invasions. For example in German waters about one half of the introduced species arrived through the hulls of sea trade ships (Nehring 2005). But since 1970 ships hulls and ballast water have similar importance to invasion processes (Nehring 2001)

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The pronounced lack of gene flow among populations might be due mostly to the fact that *Rhithropanopeus harrisi* presents a larval retention mechanism which places the larvae within the vicinity of the parental population (Cronin 1982, Cronin & Forward 1986) diminishing the probability of being taken by ships ballast tanks or of

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