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Mitochondrial DNA variability in Spanish populations of *A. italicus* inferred from the analysis of a COI region

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ABSTRACT

Key-words:
Austropotamobius italicus, *mtDNA, Cytochrome Oxidase Subunit I (COI), genetic variability, genetic structure, haplotype, conservation*

Austropotamobius italicus was once widely distributed throughout most of the country's limestone basins in Spain. But its populations have shown a very strong decline over the last thirty years, due to different factors. Thus, the species now enjoys protection under regional, national and international legislation. Therefore, knowledge of the levels and patterns of distribution of genetic diversity in crayfish populations is critical when making conservation management decisions. In the present work, the current genetic structure of Spanish populations of white-clawed crayfish, *A. italicus,* was analyzed. Eleven Spanish populations and an Italian sample were studied through an 1184 bp-lentgh sequence of cytochrome oxidase subunit I mitochondrial gene. Data analysis revealed the existence of eight haplotypes in the Iberian Peninsula, the highest diversity reported to date in Spanish crayfish. Also a substantial genetic differentiation among populations was found, with a clear geographic pattern. The genetic variability found in these populations is similar to, and even higher, than that reported in previous studies on other Spanish and European populations of *A. italicus*. Thus, given the current risk status of the species across its range, this variability in certain populations offers some hope for the species from a management point of view.

RÉSUMÉ

La variabilité de l'ADN mitochondrial des populations espagnoles d' *A. italicus* déduite de l'analyse d'une région COI

Mots-clés :
Austropotamobius italicus, *mtDNA,*

Austropotamobius italicus était autrefois largement distribué dans la plupart des régions des bassins calcaires en Espagne. Mais ces populations ont montré une baisse très forte au cours des trente dernières années, en raison de différents facteurs. Ainsi, l'espèce bénéficie aujourd'hui d'une protection législative régionale, nationale et internationale. Par conséquent, la connaissance des niveaux et des schémas de distribution de la diversité des ressources génétiques dans les populations d'écrevisses est essentielle pour prendre des décisions de bioconservation. Dans le présent travail, la structure génétique actuelle des populations espagnoles de l'écrevisse à pattes blanches, *A. italicus,* a été analysée. Onze populations

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Cytochrome Oxydase Subunit I (COI), variabilité génétique, structure génétique, haplotype, conservation

espagnoles et un échantillon italien ont été étudiés au moyen d'une longue séquence 1184 bp du cytochrome oxydase sous-unité I du gène mitochondrial. L'analyse des données a révélé l'existence de huit haplotypes dans la péninsule ibérique, la plus grande diversité signalée à ce jour dans les écrevisses espagnoles. Ainsi une différenciation génétique importante parmi les populations a été trouvée, avec une claire répartition géographique. La variabilité génétique dans ces populations est similaire, et même plus, que celle rapportée dans les études antérieures sur d'autres populations espagnoles et européennes d'*A. italicus*. Ainsi, compte tenu de la situation à risque actuelle de l'espèce dans son aire de répartition, cette variabilité dans certaines populations offre un peu d'espoir pour ces espèces d'un point de vue de bioconservation.

INTRODUCTION

Austropotamobius pallipes s. lato (Lereboullet, 1858), the white-clawed crayfish, is endemic to west and southern Europe. This crayfish plays a significant role in the dynamics of aquatic ecosystems where it inhabits, becoming, in many cases, the main biomass of the macroinvertebrates fauna. The species colonises a wide range of waterbodies, including streams, rivers, reservoirs and water-filled quarries.

This crayfish was once widely distributed and very abundant in Spain, except in the more western areas, the highest mountain ranges and the sub-desert areas of the southeast and River Ebro valley. Its populations have shown a very strong decline over the last thirty years due to different crayfish plagues, the spread of the red swamp and signal crayfishes, habitat loss and other anthropogenic impacts (Callejas *et al*., 2009). At present, the Iberian Peninsula is probably the most severely affected regression area of Europe, with a current trend of extinction ranging from 30 to 50% every five years (Alonso *et al*., 2000). Only around 500–600 small populations now remain (Alonso, 2004) occupying marginal areas or short stretches of watercourses usually isolated from the main Spanish river systems (Martinez *et al*., 2003). As consequence, the species now enjoys protection under regional, national (Spanish Ministry of the Environment and the Rural and Marine Environments, 2007) and international legislation. It is classified as vulnerable in the IUCN red list of threatened animals, and is recognised as requiring special conservation measures by different European Habitat Directives (92/43/EEC and 94/62/EU). Recovery plans are being drafted and indeed implemented by some regional authorities (Catálogo Nacional de Especies Amenazadas, National Catalogue of Endangered Species, R.D. 439/1990; INV/42). Several authors ascribed classically the Spanish populations of white – clawed crayfish as either specific, *A. pallipes,* or subspecific status of *A. italicus lusitanicus*. Recent molecular systematic investigations using mitochondrial DNA sequences have helped to elucidate some relationships within this species. Data based on 16SrRNA gene (Grandjean *et al*., 2000, 2002) have revealed that *Austopotamobius* is a species – complex that comprises three species: *A. torrentium* (mainly in Central Europe), *A. pallipes* (France, Switzerland, Germany and British Isles) and *A. italicus* (Spain, Italy, Southern Alps and Balkans). Given its wider acceptance, *A. italicus* will be the taxonomic designation used in the present paper for the Spanish population of the white – clawed crayfish.

Given the clear evidence of the animal mitochondrial genome to be an excellent target for genetic analysis (Saccone *et al*., 1999), different authors have explored its utility to reveal phylogenetic and phylogeographic relationships within the European freshwater species – complex of *Austropotamobius*. These studies included a few Iberian populations with small sample sizes from a narrow geographic area (Grandjean *et al*., 2001; Trontelj *et al*., 2005) and revealed no genetic diversity in the analyzed samples, as well as the existence of shared haplotypes between the Spanish and north western Italian populations. Surprisingly, latest reports showed the existence of genetic variability in the Iberian Peninsula populations of this crayfish species with a geographical pattern of distribution (Beroiz *et al*., 2008; Diéguez-Uribeondo, 2008; Callejas *et al*., 2009; Pedraza-Lara *et al*., 2010).

There is no compelling *a priori* reason to focus the analysis on a specific mitochondrial gene, but the cytochrome oxidase I gene (COI) does have two important advantages for phylogeographic analysis: the existence of universal primers for this gene and a rate of molecular evolution that is about three times greater than that of 12S or 16S rDNA (Knowlton and Weigt, 1998). Moreover, different regions of this gene evolve at different rates, and the patterns of sequence variability seem associated with functional constraint of the protein (Lunt *et al*., 1996). As consequence, the COOH-terminal was found to be significantly more variable than the central region of COI, loops or transmembrane helices. Furthermore, several authors pointed out that mtDNA COI gene is a powerful marker to study genetic variation at the intraspecific level in crayfish (Versteegen and Lawler, 1997; Schull *et al*., 2005) and other crustaceans (Meyran *et al*., 1997; Meyran and Taberlet, 1998; Haye *et al*., 2004).

Taking into account the above, the aims of the present work were: the study of the current genetic structure of Spanish populations of white-clawed crayfish through the use of a fragment of mitochondrial COI gene – larger that those previously used –, and propose, based on the results, areas for conservation of *A. italicus* in Spain.

MATERIAL AND METHODS

> SAMPLES

A total of 120 individuals of *A. italicus* were collected from eleven Spanish locations belonging to the main drainages throughout its distribution range and from a population of the Italian distribution area of *A. italicus*, analyzed as outgroup (Figure 1; Table I).

> DNA ISOLATION

Genomic DNA was extracted from 20–50 mg of claw muscle or periopod tissues (without killing the animal) using the DNeasy Blood and Tissue Kit from Quiagen (Valencia, CA, USA) and resuspended in Tris-EDTA (10 mM; 1 mM; pH 8.0).

DNA concentration and purity were estimated by absorbance at 260–280 nm in a NanoDrop® ND-100 (NanoDrop Technologies, Willmintong, USA) spectrophotometer. Its integrity was verified by 0.8% agarose gels in Tris-EDTA buffer (10 mM; 1 mM; pH 8). Gels were stained with ethidium bromide (1 $\mu g \cdot mL^{-1}$) and visualized with UV light transiluminator.

> COI AMPLIFICATION AND SEQUENCING

A fragment from the mtDNA COI gene was amplified in a final volume of 50 μL with 25 ng of total DNA, 1× reaction buffer, 2 mM $MgSO_4$, 200 μM of each dNTP, 15 μg of BSA, 1 μM of each primer and 1U of Vent DNA polymerase (New England Biolabs, Ipswich, MA, USA). The primers used were COI Scylla (5' TTAAGTCCTAGAAAATGTTGRGGGA, Gopurenko *et al*., 1999) and LCO (5' GTCAACAAATCATAAAGATATTGG, Folmer *et al*., 1994).

The optimal PCR programme included an initial denaturation step of 94 °C for 5 min followed by 44 cycles of 94 °C for 45 s, 53 °C for 1 min and 72 °C for 1 min 30 s, and a final extension step of 72 °C for 10 min.

Double-stranded amplified products were purified using the High Pure PCR Product Purification Kit (Boehringer-Manheim) and used as templates for sequencing reactions. These reactions were carried out with the "BIG Dye® Terminator Cycle Sequencing Ready Reaction Kit" (Applied Biosystems, Inc., USA) on a 3730 DNA Analyzer (Applied Biosystems, Inc., USA), using the primers employed for the amplification step in the Genomic Unit of the Complutense University of Madrid. The sequences of the haplotypes reported in this paper

Figure 1
Sampling locations of Spanish white-clawed crayfish included in this study. Details for each population are reported in Table I.

Figure 1
Les sites d'échantillonnage des écrevisses à pattes blanches espagnoles de cette étude. Les détails pour chaque population sont rapportés dans le tableau I.

have been deposited in the GenBank nucleotide sequence database with the accession numbers EF485041, FJ897840–FJ897842, FJ897845, JF430568, JF430569 and JF430572.

> DNA ALIGNMENT AND SEQUENCE ANALYSIS

The nucleotide sequences were aligned using CLUSTAL W software (Thompson *et al*., 1994) and edited with BioEdit v 7.0.9.0 (Hall, 1999). After alignment and edition, the final sequence length used was 1184 bp. Translation of the nucleotide sequences were performed through SeqBuilder programme (DNASTAR software package, DNASTAR, Inc., 2004). The genetic diversity estimates (haplotype diversity, H; nucleotide diversity, π, number of segregating sites, S) were calculated using DnaSP v 4.50.3 programme (Rozas *et al*., 2008).

A Median-Joining network was carried out to determine the relationships among the haplotypes found. This approach is the most suitable to establish connexions among closely related sequences (Cassens *et al*., 2003) (NETWORK v. 3.1.1.1, http://www.flexus-engineering.com). The COI haplotype frequencies were geographically depicted for each population using PhyloGeoViz v 2.4.4 (Tsai, 2010).

Exact test of population differentiation (Raymond and Rousset, 1995) was used to check the null hypothesis that observed haplotype distribution is random with respect

Table I

Samples of A. italicus *(10 individuals per population) studied in the present work. Columns show respectively: code, population, watershed and drainage direction, locality and region, haplotypes found, number of polymorphic sites (S), haplotype diversity (Hd) and nucleotide diversity (π).*

Tableau I

Les échantillons d'*A. italicus* (10 individus par population) étudiés dans le présent travail. Les colonnes représentent respectivement : le code, la population, les bassins versants et la direction d écoulement, la localité et la région, les haplotypes trouvés, nombre de sites polymorphes (S), la diversité des haplotypes (HD) et la diversité nucléotidique (π).

	Code	Population	Watershed/Drainage direction	Collection sites	Haplotypes found	S	H_d	π
1	**AME**	Arroyo Mestas	Sella/Cantabric (North)	Cangas de Onís/Asturias	2	0	0	0
2	**AZU**	Río Azumara	Miño/Atlantic (West)	Castro de Rei/Lugo	1	0	0	0
3	**CUE**	Arroyo Cuende	Guadiana/Atlantic (West)	Huerta Obispalia/Cuenca	3, 6	1	0.20	0.00017
4	**GIR**	Arroyo Santa Margarida	Costero Catalana/Mediterranean (East)	Olot/Gerona	1, 8	1	0.20	0.00017
5	**GRA**	Arroyo Ermitas	Guadalquivir/Atlantic (West)	Albuñuelas/Granada	1	0	0	0
6	**LRC**	Lago Ercina	Sella/Cantabric (North)	Cangas de Onís/Asturias	2	0	0	0
7	**MAD**	Manantial Madalena	Duero/Atlantic (West)	Rebolledo de Traspeña/Burgos	2	0	0	0
8	**NAV**	Río Ega	Ebro/Mediterranean (East)	Estella/Navarra	4, 7	1	0.20	0.00017
9	**POZ**	Arroyo Pozuelo	Tajo/Atlantic (West)	El Pozuelo/Cuenca	3	0	0	0
10	**PVS**	Río Nervión	Nervión/Cantabric (North)	Altuve/Álava	4	0	0	0
11	**SEN**	Barranco del Salt	Júcar/Mediterranean (East)	La Pobla de Benifassà/Castellón	1, 3, 5	2	0.64	0.00092
12	**ITA**	Río Bisenzio	Arno/Ligurian	Prato/Italy	1	1	0	0

to sampling location. The Ewens – Watterson neutrality test with 10 000 permutations was also ran (ARLEQUIN v3.11).

An Analysis of Molecular Variance (Excoffier *et al*., 2005) was performed to estimate the variance components, in order to assess the partitioning of genetic variation among and within the populations sequenced, as well as, among the watershed sampled. The levels for these variance components were calculated using permutational procedures.

Since none of the genetic distances stand out as best in all situations (models of mutation, effective population size, population size reduction, etc.), genetic differentiation was estimated in two ways. Firstly, from Nei's genetic distances (Dxy) (1987) based on the average number of pairwise nucleotide substitutions per site between populations. Secondly from Fst values (Hudson *et al*., 1992), this quantifies how genetic diversity is partitioned within and between populations. In addition, gene flow (Nm) was calculated from Fst indicator. Genetic distances were obtained using DnaSP v 4.50.3 software package (Rozas *et al*., 2008). Both matrixes were compared by a randomized test for matrix correspondence – the Mantel test (Mantel, 1967) – to check correlation between both genetic distances calculated.

Principal component analysis (PCA) (Sneath and Sokal, 1973) was performing using NTSYSpc v2.10q software package (Rohlf, 2000) to visualize the grouping populations.

Finally, correlation between genetic and geographic distances was analyzed employing the Mantel test (Mantel, 1967) through IBD program (Jensen *et al*., 2005) to check for patterns of isolation by distance. Geographical distances between populations were calculated from their latitude and longitude, using an online geographical distance calculator (http://www.cactus2000.de/uk/unit/massgrk.shtml).

RESULTS

Nucleotide sequences of 1184 pb in length were obtained from the COI gene of the 120 specimens, the longest for this species reported to date. The fragment amplified comprises the complete gene excepting 350 bp, and corresponds to positions 1522–2705 of the *Drosophila yakuba* (Clary and Wolstenholme, 1985) and positions 52–1242 of the *Cherax destructor* (AY383557, Miller *et al*., 2004) mtDNA sequences. The average A + T content for these sequences was 0.6266.

Sequence analysis among 110 individuals of *A. italicus* from 11 Spanish populations and 10 individual from the Italian sample revealed 7 SNPs. Four out seven SNPs were informative under parsimony and two, produced a shift in the amino acid sequence of the protein encoded by this gene. Differences between haplotypes, as nucleotide substitutions, ranged between 1–4 mutations. (0.084–0.338%) (Table II).

Eight haplotypes were identified, four of them at intermediate frequencies (Haplotypes 1–4). The remaining four, at low frequencies, were private of those populations in which were located. As a whole, 90 percent of specimens had one of the four main haplotypes. Likewise, Haplotypes 1 and 2 account for about 55% of individuals, differing in two transitions in positions 702 and 1002. Both constitute the two most common haplotypes in Spain. The Italian sampled analysed was monomorphic for Haplotype 1.

The species *A. italicus* show a haplotype diversity of 0.766 and a nucleotide diversity of 0.00111 in the Iberian Peninsula. Regarding the populations, seven out of eleven hold genetic diversity at COI mtDNA gene level. The highest haplotype and nucleotide diversity were found in the population encoded as SEN, where three different haplotypes were detected (1, 3 and 5). The populations CUE, GIR and NAV showed two different haplotypes each one. The remaining 7 Spanish populations were monomorphic but for different haplotypes while individuals of the Italian sample held a single haplotype (Haplotype 1), one of the most common in the Iberian Peninsula (Table I).

The network generated by the Median Joining (MJ) method revealed the relationships among haplotypes. All of them were closely related and had neither intermediate nodes nor haplotypes not sampled. Moreover, the MJ network designated Haplotype 1, one of two most

Table II

Haplotypes found in A. italicus *Spanish populations. Columns 2–8 refer to position of the SNP in the 1184 nt sequence, Freq.: frequency of each haplotype in the total of individuals analyzed, Nu. Indiv.: number of individuals represented by each haplotype, Nu. pop.: number of populations in which they were detected. Bold numbers: SNP informative under parsimony. ♦ Produce an aminoacid change.*

Tableau II

Haplotypes dans les populations espagnoles *d'A. italicus*. Colonnes 2–8 : référence à la position de la SNP dans la séquence de 1184 nt, Freq. : fréquence de chaque haplotype dans le total des individus analysés, Nu. Indiv. : nombre d'individus représentés par chaque haplotype, Nu. pop. : nombre de populations dans lesquelles ils ont été détectés. Les chiffres en gras : SNP informatif sous parcimonie. ♦ Produit un changement d'acide aminé.

	96	219	634	702	754	968	1002	Freq.	Nu. Indiv.	Nu. Pop.
Haplotype 1	C	C	T	C	A	T	A	27.27	30	4
Haplotype 2	C	C	T	T	A	T	G	27.27	30	3
Haplotype 3	C	T	T	C	A	T	A	21.82	24	3
Haplotype 4	C	C	T	C	A	T	G	17.27	19	2
Haplotype 5	C	C	T	C	A	C	A	3.64	4	1
Haplotype 6	T	T	T	C	A	T	A	0.91	1	1
Haplotype 7	C	C	T	C	C ♦	T	G	0.91	1	1
Haplotype 8	C	C	C	C	A	T	G ♦	0.91	1	1

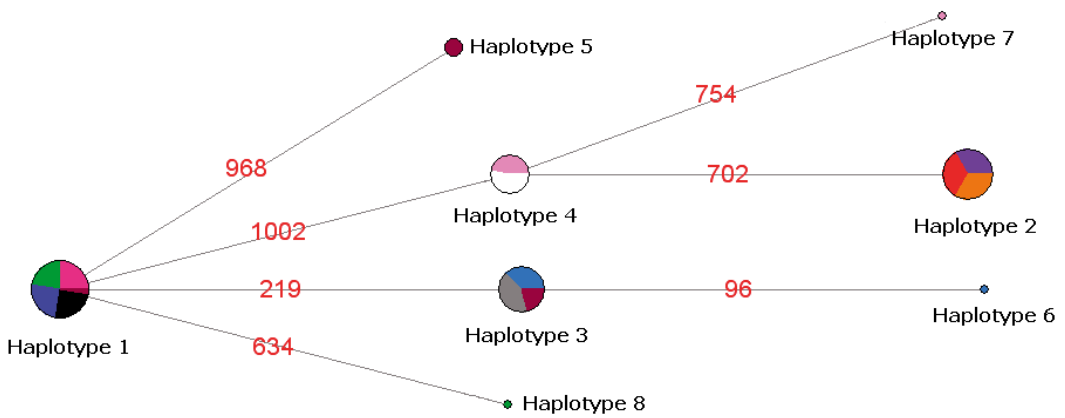

Figure 2

Haplotypes network of the COI gene fragment generated by "Median-Joining" method. Red numbers indicate the mutation positions in the 1184 nucleotides analyzed.

Figure 2

Réseau haplotypes du fragment du gène COI générés par la méthode "médian-joining". Les numéros rouges indiquent les positions des mutations dans les 1184 nucléotides analysés.

common, as the ancestral one (Figure 2). From a single to four mutational steps were needed to connect the eight haplotypes.

The results of exact test of population differentiation, based on haplotype frequencies, revealed statistically heterogeneity in the distribution of haplotypes across samples ($p < 0.05$). Genetic distances values between populations obtained from Dxy (Nei, 1987) and Fst (Hudson *et al*., 1992) correlated significantly across comparisons (Mantel test, $r = 0.7035$, $p < 0.001$, in 1000 permutations). Both procedures revealed significant values in comparisons between populations, except for those populations monomorphic for the same haplotype (Table I, Table III). The highest Fst distances were found between those populations monomorphic for different haplotypes.

As a whole, Fst value revealed significant population differentiation, at the mtDNA COI gene, for this crayfish species in Spain (Fst = 0.89702, $p < 0.001$, based on 1000 permutations).

Table III

Fst pairwise genetic distances for the Spanish populations studied. Non-significant values at $p < 0.05$ are indicated with an asterik.

Tableau III

Distances génétiques FST pour les populations espagnoles étudiées. Les valeurs non-significatives à $p < 0{,}05$ sont indiquées par un astérisque.

	AME	AZU	CUE	GIR	GRA	LRC	MAD	NAV	POZ	PVS
AZU	1.00000									
CUE	0.96774	0.90909								
GIR	0.95238	0.0000*	0.83333							
GRA	1.00000	0.0000*	0.90909	0.0000*						
LRC	0.0000*	1.00000	0.96774	0.95238	1.00000					
MAD	0.0000*	1.00000	0.96774	0.95238	1.00000	0.0000*				
NAV	0.90909	0.90909	0.90909	0.83333	0.90909	0.90909	0.90909			
POZ	1.00000	1.00000	0.0000*	0.90909	1.00000	1.00000	1.00000	0.95238		
PVS	1.00000	1.00000	0.95238	0.90909	1.00000	1.00000	1.00000	0.0000*	1.00000	
SEN	0.81226	0.39506	0.35556*	0.35556	0.39506	0.81226	0.81226	0.67778	0.39506	0.71345

Table IV

AMOVA analysis of the 110 individuals from all 11 Spanish populations of white-clawed crayfish using COI sequences. The data show the degrees of freedom (df), percentage of total variance contributed by each component, and the probability (p) of obtaining a more extreme component by chance alone. 1000 permutations were used for analysis.

Tableau IV

Analyse AMOVA des 110 individus des 11 populations espagnoles d'écrevisses à pattes blanches utilisant des séquences COI. Les données montrent les degrés de liberté (df), le pourcentage de la variance totale apportée à chaque composante, et la probabilité (*p*) d'obtenir une composante plus extrême par le seul hasard. 1000 permutations ont été utilisées pour l'analyse.

Source of variation	df	Variance component	Percentage total variance	*p*-value
All populations: 1 group				
Among populations	10	67.636	89.70	< 0.001
Within populations	99	7.600	10.30	< 0.001

There was evidence for isolation by distance (Mantel test: $r = 0.6006$, $p < 0.0010$). The inferred Nm value was 0.06.

The AMOVA analyses (Table IV) showed that genetic variation expressed among populations was 89.70%, whereas about a 13.5% occurred within samples. A random permutational test revealed that only watershed and sea basins were not statistically significant components.

The principal component analysis (Figure 3) assigned these populations into 4 groups coincident with the 4 most common haplotypes. The first PCA axis explains 46.53% of variance and reveals two well-separated groups: AME, LRC, MAD (mainly Haplotype 2) and AZU, GIR, GRA, ITA (mostly Haplotype 1) populations from the remaining samples. The second PCA axis explains 30.76% and disjoined populations with Haplotype 1 from those with Haplotype 2. Otherwise, about 25% of the variance was explained by the third axis and separates the other four samples (CUE, POZ, NAV and PVS) according to the main haplotype present in each population. Thus, samples with Haplotype 3 (CUE and POZ) were spitted from populations with Haplotype 4 (PVS and NAV). The population encoded as SEN did not belong to any group. Three haplotypes were detected in this sample, haplotypes 1 and 3, and also, haplotype 5 (exclusive for this population) in four out of ten individuals studied.

The allele distribution was not neutral according to Ewens – Waterson neutrality test for CUE, GIR, NAV or SEN. In the remaining populations this test could not be run, as onlyone haplotype was present. In this way, as shown in Figure 4, haplotypes found were

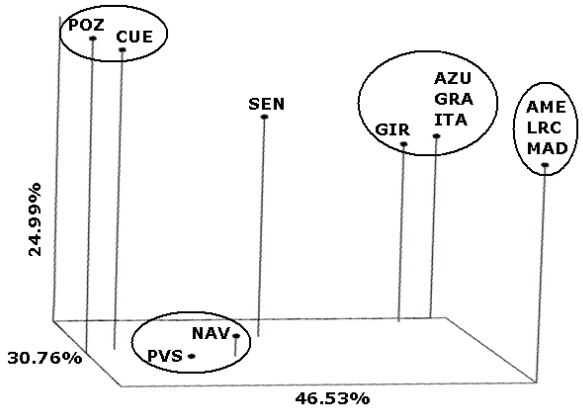

Figure 3
Results of the PCA analysis based on mtDNA COI gene sequences from different samples of A. italicus*. Eigenvalues for each principal component are listed besides each axis.*

Figure 3
Résultats de l'analyse PCA des séquences d'ADNmt du gène COI à partir d'échantillons différents *d'A. italicus*. Les valeurs propres pour chaque composante principale sont énumérées à côté de chaque axe.

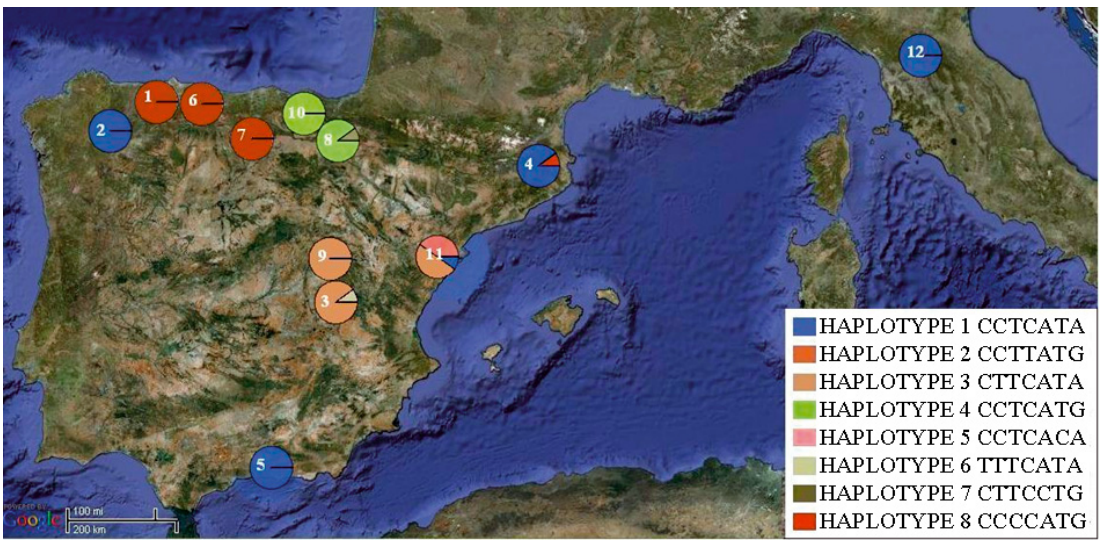

Figure 4
Geographical localization and genetic composition of 120 individuals from 12 different populations. Each population is shown as a pie chart representing membership proportions in the eight haplotypes found.

Figure 4
Localisation géographique et composition génétique de 120 individus de 12 populations différentes. Chaque population est représentée par un diagramme circulaire représentant les proportions des effectifs dans les huit haplotypes trouvés.

not evenly distributed across Spain, but instead appears restricted to particular regions. Overall, Haplotypes 1 and 3 were distributed mainly in the Mediterranean area while, Haplotypes 2 and 4 were located in the north of the Iberian Peninsula. Hence, distribution of these haplotypes presented a marked geographical pattern.

DISCUSSION

> *LEVELS OF GENETIC VARIABILITY*

An 1184b bplong COI region was amplified – the longest for this species reported to date. Sequences were obtained from eleven Spanish populations belonging to ten watersheds and from an Italian sample analyzed as outgroup (Table I, Figure 1).

In the present work eight haplotypes are described based on seven SNPs found, four of them informative under parsimony. Haplotypes found in the Iberian Peninsula are closely related (Figure 2) since from a single to four mutational steps are needed to connect the eight haplotypes and there are not intermediate nodes. The MJ network designate to Haplotype 1 as the ancestral one. This haplotype show the widest distribution range and in addition, it is one of the most common in Spain and also present in the Italian sample. The great majority of the nucleotide differences are transitions and only two amino acid changes are observed.

The present results show that 1184 bp-length COI fragment is more sensitive for detecting genetic variability in Spanish populations of the threatened white – clawed crayfish than others previously used. Our results indicate that the species *A. italicus* had a haplotype diversity of 0.766 and a nucleotide diversity of 0.00111 in the Iberian Peninsula. The degree of genetic diversity (Hd, π, Table I) is higher than previously reported for Iberian crayfish (Grandjean *et al*., 2001; Trontelj *et al*., 2005; Diéguez-Uribeondo *et al*., 2008; Pedraza-Lara *et al*., 2010) and similar to values obtained for European populations of *A. italicus* (Zaccara *et al*., 2005; Baric *et al*., 2006).

The absence of genetic variability in Spanish populations and their close relationships with northern Italian samples, found in previous studies, triggered the debate over whether the Iberian Peninsula has been artificially stoked (Albrecht, 1982; Laurent, 1988). However, our outcomes do not support this hypothesis because four out of eleven Spanish populations analyzed in this work show genetic variability. These samples – CUE, GIR, NAV, SEN – come from large populations at restricted or protected areas where disease or overfishing have been, in part, avoided and its effective population numbers remain at high levels. It is necessary to notice that different analyses carried out by our group with these same populations are consistent with the present results. For instance, the GRA sample, one of the most southerly of Europe, show low degree of genetic variability at mtDNA COI gene level, RAPDs, ISSR (Beroiz *et al*., 2008; Callejas *et al*., 2009), as well as microsatellite analysis (Matallanas *et al*., submitted). This population was once large but, owing to disease caused by the fungus *Saprolenia parasitica* (among other factors), its numbers crashed during the 1990s (Gil and Alba-Tercedor, 1998, 2000). Otherwise, NAV is one out of four populations that hold haplotype diversity at mitochondrial level, likewise showed the highest polymorphism values by RAPDs, ISSRs or SSRs (Beroiz *et al*., 2008; Callejas *et al*., 2009; Matallanas *et al*., submitted). This population is located in a protected area as was mentioned above.

Genetic variability is not found within eight samples studied in the present work including in the Italian one analyzed as outgroup, although several of them are monomorphic for different haplotypes. Decline of crayfish populations due to several factors such as loss of habitat, pollution, overfishing or crayfish plague transmitted by introduced species has been observed in Spain over the last thirty years (Torre Cervigon and Rodríguez Marques, 1964; Diéguez-Uribeondo *et al*., 1997; Galindo *et al*., 2003; Rallo *et al*., 2004). Genetic drift and inbreeding in small-effective size populations lead to the loss of genetic variability and probably contribute to our results.

PATTERNS OF GENETIC VARIABILITY

Sequence analysis of the COI fragment used show a significant degree of genetic differentiation among the Spanish populations studied. The analyses of molecular variance (Table IV) indicate that most of the genetic variation found was among populations. However, no important levels of genetic differentiation are seen among hydrological or ocean (*i.e.* Mediterranean

and Atlantic) basins. Otherwise, PCA analysis reveals that samples are grouped according to the main haplotype present in each population (Figure 3).

The high Fst value (89.70%) and genetic distances (Table III) also indicate differentiation among populations. The highest Fst distances are found between those populations monomorphic for different haplotypes, such as comparisons between AME, LRC or MAD (Haplotype 2) and AZU or GRA (Haplotype 1). There are evidences for isolation by distance in Iberian populations (Mantel test, $r = 0.6006$, $p < 0.0010$). These agree with data reported by other authors for both Spanish and European white – clawed crayfish (Gouin *et al*., 2001; Callejas *et al*., 2009; Pedraza-Lara *et al*., 2010).

The existence of small and isolated populations leads to divergence between populations and homogeneity within them. Conversely, the presence of large and connected populations results in less differentiation among them and higher diversity within them (Lin *et al*., 1999). The gene flow estimated for the Spanish populations of crayfish was very low, 0.06. Thus, the current structure and recent history of the Spanish populations of *A. italicus* was probably shaped by the joint action of bottlenecks and genetic drift. However, ancient historical events such as population fragmentation, recolonizations from refugia during the ice ages (Grandjean and Souty-Grosset, 2000; Grandjean *et al*., 2001; Gouin *et al*., 2003; Trontelj *et al*., 2005; Diéguez-Uribeondo *et al*., 2008), the formation of fluvial basins, must also have influenced their present structure, as well as it has been demonstrated in other species (Hewitt, 1996, 2001; Callejas and Ochando, 2002). Besides, it should be noted that human translocation of crayfish among different regions of Spain has been common practice since 19th century (Pardo, 1942; Torre Cervigon and Rodríguez Marques, 1964) and probably, it also influenced the current genetic population structure of this crayfish.

Haplotypes found in the Iberian Peninsula show a clear pattern (Figure 4). One out of eight haplotypes found in this study is shared by the Spanish and Italian populations (Haplotype 1) whereas the remaining seven are exclusive for the Spanish locations (Table II). Haplotypes 2 and 4 are located in Northern Spain. It is interesting to note that Haplotype 4 is only found in populations NAV and PVS, geographically very close, although both belonging to different watersheds. The same is true for Haplotype 2. On the contrary, Haplotypes 1 and 3 are present in the Mediterranean area, although Haplotype 1 shows a wider distribution area than Haplotype 3. Additionally, most of the private haplotypes (Haplotype 5, 7 and 8) found in this study belong to the Mediterranean (East) basin.

The amplified fragment made possible to detect a relevant mtDNA pattern in the Iberian Peninsula crayfish populations. This genetic structure appears clearer than in previous reports (Diéguez-Uribeondo *et al*., 2008; Pedraza-Lara *et al*., 2010), although their outcomes fit into the structure highlighted in the present work.

Our data do not seem to support the assumption of an anthropogenic origin of the white-clawed crayfish in Spain from the North of Italy at 19th century (Grandjean *et al*., 2001; Trontelj *et al*., 2005). If Spanish and Italian populations derived from an older Mediterranean population – whose distribution range decreased during the Miocene (Karaman *et al*., 1963) –, the relationship between them could be explained. Furthermore, genetic drift effect and bottlenecks during and after glaciations would lead to the loss of less frequent alleles while the most common haplotypes would be present in both areas, such as Haplotype 1. This common origin also could explain the relationship found between populations belonging to these two countries (Diéguez-Uribeondo *et al*., 2008). Otherwise, estimated mutation rate in crustacean mtDNA is around 2.75% per million years (Wares and Cunningham, 2001). Thus, it is unlikely that genetic variability observed in Spanish populations could be explained after a founder effect due to translocations during the 19th century as was proposed by some authors (Grandjean *et al*., 2001; Trontelj *et al*., 2005).

In conclusion, the present results show that the 1184 bp-lentgh sequence of mtDNA COI gene studied is a sensitive tool for assessing the genetic diversity and structure of the Spanish populations of *A. italicus*. The work has already revealed the existence of eight mtDNA haplotypes, the highest diversity reported to date in Spanish crayfish and a substantial genetic differentiation among populations with a clear geographic pattern. Thus, given the current

risk status of the species across its range, this variability in certain populations offers some hope for the species from a management point of view. Knowledge of the levels and patterns of distribution of genetic diversity – the basis for viability and future evolution of populations (Avise, 2000; Moritz *et al*., 2002) – is critical when making conservation management decisions because the loss of genetic variation put wildlife populations at an increased risk (Reed and Frankham, 2003). In this context, four populations should be specially considered in future recovery programmes: Huerta de Obispalia (CUE), Olot (GIR), Estella (NAV) and La Pobla de Benifassà (SEN). These populations hold seven out of eight haplotypes found. Moreover, Cangas de Onís (AME and LCR) could be a critical area for conservation since it contains one of the most common haplotypes in Spain but absent in the populations mentioned above.

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