GENETIC ANALYSIS FOR CONSERVATION OF *AUSTROPOTAMOBIUS ITALICUS* POPULATIONS IN MARCHES REGION (CENTRAL ITALY)

R. CATAUDELLA (1), N. PUILLANDRE (2), F. GRANDJEAN (2)

(1) Istituto di Scienze Morfologiche, Università di Urbino "Carlo Bo", Campus scientifico, Località Crocicchia, I-61029 Urbino PU, Italy. E-Mail: s.cataudella@uniurb.it

(2) Laboratoire de Génétique et Biologie des Populations de Crustacés, UMR 6556, Université de Poitiers, 40, avenue du Recteur Pineau, F-86022 Poitiers cedex, France.

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ABSTRACT

The genetic characteristics of white-clawed crayfish populations in Marches Region (Central Italy) was investigated to plan conservation strategies. In order to clarify their taxonomic status, 50 specimens from ten populations were collected in six different hydrographic drainages of the Umbro-Marchigiano Appennine. The genetic relationships of the *Austropotamobius italicus* specimens were assessed using DNA sequences of the mitochondrial DNA 16S rRNA gene.The 3 distinct haplotypes M1, M2, M3 detected have been compared to those available in GenBank corresponding to four subspecies described for *A. italicus*. Our results confirmed the presence of both subspecies *A. i. carsicus* (M1 & M2) and *A. i. meridionalis* (M3) in Marches region. Two mixed populations with both subspecies have been characterized. These new data of the genetic structure of population will be used to plan management and recovery programs.

Key-words: *Austropotamobius italicus*, subspecies, taxonomy, conservation.

ANALYSES GÉNÉTIQUES POUR LA CONSERVATION DES POPULATIONS D'*AUSTROPOTAMOBIUS ITALICUS* DANS LA RÉGION DES MARCHES (ITALIE CENTRALE)

RÉSUMÉ

Les caractéristiques génétiques des populations d'écrevisses à pattes blanches de la région des Marches (Italie centrale) ont été étudiées dans le but de définir une stratégie de conservation. 50 individus provenant de 10 populations appartenant à six bassins hydrographiques des Apennins, de l'Ombrie et des Marches ont été prélevés dans le but de déterminer leurs statuts taxonomiques. Les relations génétiques entre individus d'*Austropotamobius italicus* ont été appréhendées par sequençage d'une portion du gène d'ADN mitochondrial codant pour l'ARN ribosomique 16S. Les trois haplotypes obtenus, M1, M2 et M3, ont été comparés à ceux mis en ligne dans GenBank correspondant aux quatre sous-espèces décrites chez *A. italicus*. Nos résultats confirment la présence dans la région des deux sous-espèces *A. i. carsicus* (M1 & M2) et *A. i. meridionalis* (M3). Deux populations mixtes ont été caractérisées. Ces nouvelles données sur la structure

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génétique des populations seront utilisées pour définir des plans de gestion et pour mettre en œuvre des programmes de repeuplement.

Mots-clés: *Austropotamobius italicus,* sous-espèces, taxonomie, conservation.

INTRODUCTION

The white-clawed crayfish, *Austropotamobius pallipes* complex, is widespread throughout Europe and its range has been drastically restricted (HOLDICH, 2002) due to a combination of several factors affecting the habitat quality or the health of populations (FÜREDER *et al*., 2003). To protect the existing populations, numerous conservation plans thoughout Europe have emerged during the last 10 years, often including genetic analysis of populations to help management decisions. These studies have revealed a high genetic structure among European populations and more particularly among Italian ones (SANTUCCI *et al*, 1997; LÖRTSCHER, 1998; GRANDJEAN *et al*., 2000; LARGIADER *et al*., 2000, FRATINI *et al*., 2005, TRONTELJ *et al*., 2005; ZACCARA *et al*., 2004 and 2005). These authors reported the presence of two well differentiated groups in Europe corresponding to *pallipes* and *italicus* specimens. Although discussions on the status of these two groups are still in progress, separate species status for *pallipes* and *italicus* was originally proposed by GRANDJEAN *et al*. (2002) and accumulating evidence favours that interpretation. Within *italicus* species, these studies showed a strong genetic structure with the presence of four differentiated groups (FRATINI *et al*., 2005): *A. i. italicus* in the Tuscan-Emilian Apennine (i.e. central Italy); *A. i. carsicus* in North-eastern Italy; *A. i. carinthiacus* in Central and and North-western Italy and *A. i. meridionalis* corresponding to specimens from Latium, Abruzzi, and Southern Italy and also including Slovenian specimens. However, if we take in account the extremely close genetic relationship between *A. i. italicus* and *carinthiacus*, it seems to be more reliable to cluster these two subspecies into one *A. p. italicus* as proposed by Machino in HOLDICH (2002). Actually *Austropotamobius italicus* is not an official recognised taxon, but the complex phylogenetic structure of the *Austropotamobius pallipes* supports the necessity of a systematic revision.

The aim of the present study was to investigate the genetic relationships among appennine populations by the analysis of sequences of 16 S RNA gene to determine the subspecific status of specimens. This preliminary study on the genetic characteristics of populations in Marches Region will be used to plan management and recovery programs.

MATERIALS AND METHODS

We collected 50 specimens (five specimens from each population) by hand from ten different rivers of the Umbria-Marches Appennine in six hydrographic drainages of the Adriatic sea (Figure 1). One pereopod was taken per individual and was put in absolute ethanol. Tissues were dehydrated (2 min) in sterile water and ground with plastic pestles in microcentrifuge tubes containing 100 mM Tris, 10 mM EDTA, 100 mM NaCl, 0.1% SDS, 50 mM DTT and 10 µl/ml proteinase K (pH = 8). Samples were incubated at 37°C for 4 hours and DNA extracted twice with phenol/chloroform/isoamyl alcohol (25: 24: 1) followed by an additional extraction with chloroform. The DNA was then precipitated with one volume of isopropanol and 1/10 volume of 3 M (pH = 5.2) sodium acetate. It was then dried and resuspended in sterile water. DNA was collected by centrifugation, dried and diluted in water to a final concentration of 20 ng/µl.

Polymerase chain reaction (PCR) amplification of of a rDNA 16S portion about 500 base pairs (bp) long was carried out in a Progene thermocycler using primers available in GRANDJEAN *et al*. (2002).

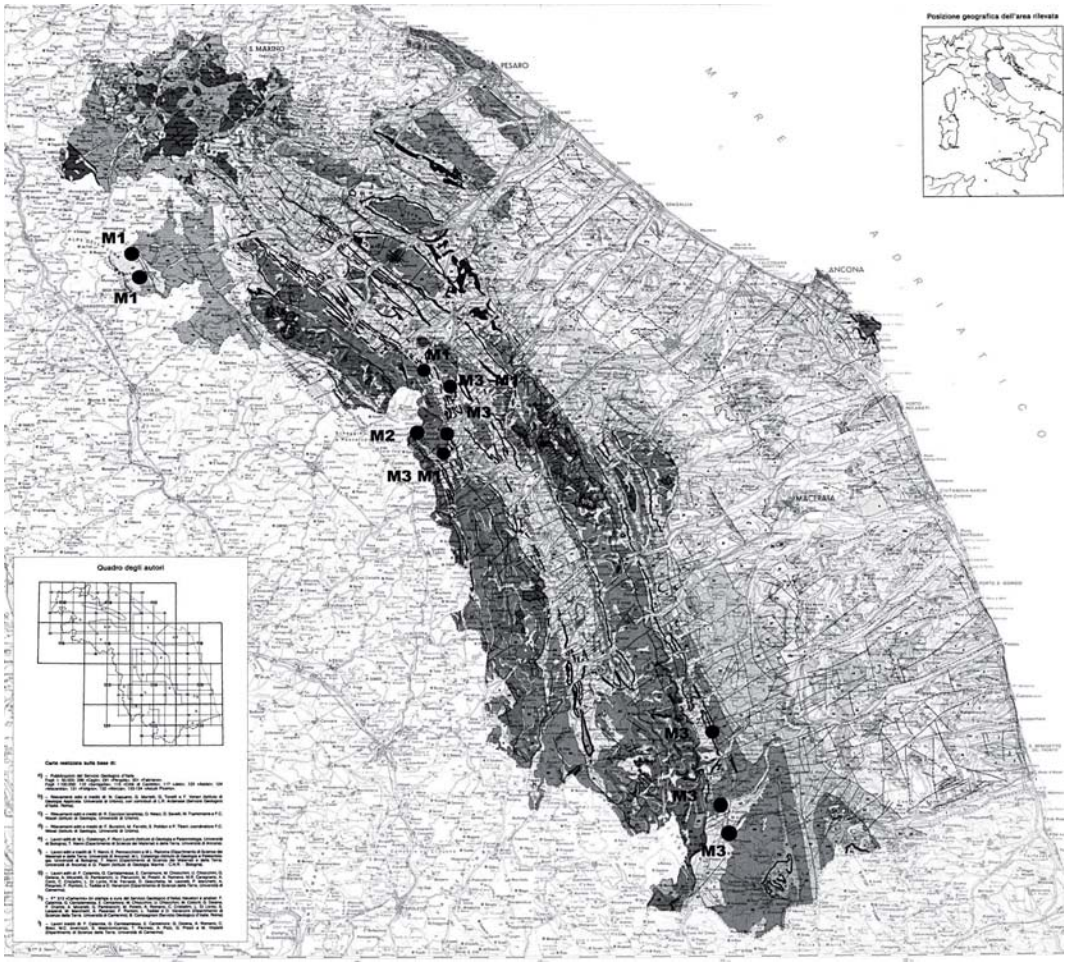


Figure 1
Geographical distribution of haplotypes of *Austropotamobius italicus* subspecies in Marches region.

Figure 1
Distribution géographique des haplotypes des sous-espèces de *Austropotamobius italicus* dans la région des Marches.

The optimal cycling program was 2 min at 94°C, 1 min at 35°C, 2 min at 72°C followed by 1 min at 94°C, 1 min at 35°C and 2 min at 72°C for 44 cycles and a final extension of 5 min at 72°C, using a Trio-Thermoblock (BIOMETRA GmBH, Göttingen, Germany).DNA Alignment-The nucleotide sequences were aligned manually with Se-Al v1.0al (Sequence Alignment Editor Version 1.0 alpha 1; Andrew Rambaut, 1996).

The sequences obtained from this study were combined with other sequences deposited in GenBank and corresponding to Italian, French and Swiss populations; a sequence of *A. torrentium* from Genbank was also used as outgroup (AF237599; GRANDJEAN *et al*., 2000a).

Neighbour-Joining (NJ), and Maximum Parsimony (MP) methods were used for phylogenetic reconstructions. The NJ and MP and analyses were conducted on MEGA version 3.0 (KUMAR *et al*., 2004). MP trees were found by heuristic search using

101 replicates of random stepwise addition. Bootstrap Percentages (BP_{MP}) were computed after 1,000 replicates. The NJ tree was found using the "p" distance. The pairwise sequence divergence values among haplotypes were used to assess the mean sequence divergence between the two main clusters revealed in phylogeographic analyses.

RESULTS

All the analyses were based on a 301 bp alignment of mitochondrial 16 S sequences. The sequences imported from GenBank were 199 bp shorter than those of 500 bp obtained in this study. The sequence alignments consisted of 301 pb enclosing 62 variable sites, of which 26 are parsimony informative. Among Marches samples three distinct haplotypes M1, M2, M3 were detected (Table I). The geographical distribution of Marches' haplotypes is presented in Figure 1. Among these three haplotypes, M1 and M2 were closely related with a nucleotide distance, pi, between the two haplotypes of 0.6%. These two haplotypes differ from M3 by a mean of 2.3%. These haplotypes show a highly structured geographical pattern of distribution (Figure 1 and Table 1). Haplotype M1 was found in northern locations whereas haplotype M3 was found in southern locations. M2 was specific to one location: Rio Freddo. Two centrally located populations harbour both M1 and M3.

Both phylogenetic analyses from NJ and MP revealed the presence of two subspecies in hydrographic basins of Marches region: *A. i. carsicus* (M1 & M2 haplotypes) that has been detected up to now only in the North-eastern Italy and in Alpi Orobie and *A. i. meridionalis* (M3) in the south. Two mixed populations with both subspecies *A. i. carsicus* and *A. i. meridionalis* were present in Amandole and Sanguerone from the hydrographic basin of Esino (the middle of Marches region) (Table I).

Pairwise mt16S sequence divergence was reported in Table II. The level of genetic variation within *A. italicus* subspecies is represented by the pairwise p-distance extimation (expressed as percentage) in Table II.

DISCUSSION

Our results showed that two subspecies overlap in Marches Region of Italy, *A. i. carsicus* and *A. i. meridionalis*. The distribution of *A. i. carsicus* in the central-northern part of the region was in accordance with the Italian ichthyogeographic Padan-Venetian (PV) district identified by BIANCO (1993). It could be explained by the extension of the Po River basin as far as the border of the meso-adriatic ditch, capturing water from a large number of rivers on both sides of the Adriatic Sea. In fact during glaciations the lowering of the sea level determined confluences of water between rivers flowing into the epicontinental area of the Mediterranean sea (CATTAUTO *et al*., 1988). *A. i. meridionalis*, recently described by FRATINI *et al*. (2005) from molecular data, and to the Apennine cluster defined by TRONTELJ *et al*., 2005 is located in Central-Southern Italy from both side of the Apennines chain. According to these authors, its presence in Slovenia could result from recolonization events from glacial refugial area during the Pleistocene due to the lowering of the sea level and the consequent confluence of some Adriatic rivers (BIANCO, 1995). In this study, we showed that its northern distribution reaches the central Marches Region where this subspecies is in contact with *A. i. carsicus* in the hydrographic basin of Esino. Two hypotheses could be drawn to explain the existence of mixed populations in central Marches region. They could be the result of a natural secondary contact between the two species during their spreading from different refugia after the last glacial period of the Pleistocene. According to the distribution of *A. i. meridionalis*, the refugial area during the last glaciation period could be located in the south of Italy, and from that area post-glacial recolonisation events had taken place towards the north. Translocations by humans

Table I

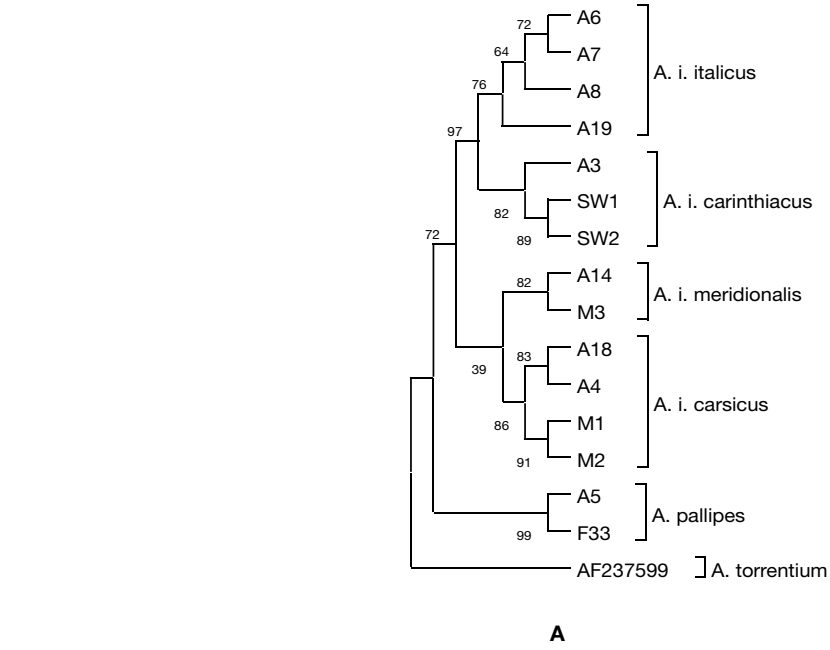
For each site, data are reported of: water body and its hydrographic drainage; region or country; sample size; mitochondrial haplotypes (in parentheses, the number of individuals for each haplotype). Some sequences are downloaded from GeneBank.

Tableau I

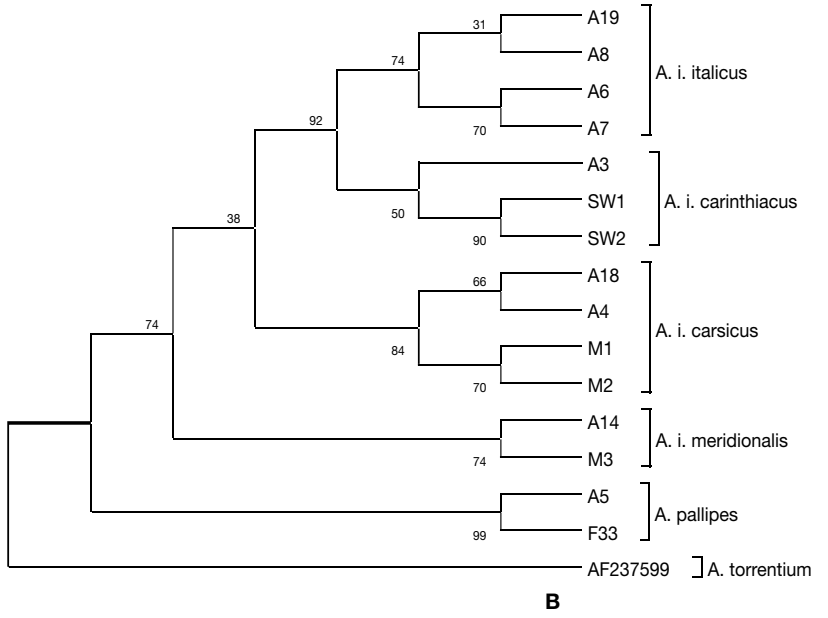
Pour chaque site, les données suivantes sont reportées: cours d'eau, bassin hydrographique, région ou pays, taille de l'échantillon, haplotypes mitochondriaux (entre parenthèses, le nombre d'individus pour chaque haplotypes). Quelques séquences ont été téléchargées à partir de GeneBank.

Code	Water body	Hydrographic drainage	Sampling location	Sample size	Haplotype 16S(n)	Genebank accession number, original source and taxonomic reference
1	Meta	Metauro	Marche	5	M1 (5)	REF genebank (present paper): *A. i. carsicus*
2	Auro	Metauro	Marche	5	M1 (5)	*A. i. carsicus*
3	Cesano	Cesano	Marche	5	M1 (5)	*A. i. carsicus*
4	Pozzatoio	Esino	Marche	5	M3 (5)	REF genebank (present paper): *A. i. meridionalis*
5	Amandole	Esino	Marche	5	M3 (2); M1(3)	*A. i. meridionalis* & *A. i. carsicus*
6	Sanguerone	Esino	Marche	5	M3 (3); M1 (2)	*A. i. meridionalis* & *A. i. carsicus*
7	Rio Freddo	Esino	Marche	5	M2 (5)	REF genebank (present paper): *A. i. carsicus*
8	Cannavì	Aso	Marche	5	M3 (5)	*A. i. meridionalis*
9	Lago	Tronto	Marche	5	M3 (5)	*A. i. meridionalis*
10	Lera	Tenna	Marche	5	M3 (5)	*A. i. meridionalis*
11	Varaita	Po	Piemonte	2	A5 (5)	AY611201 (FRATINI *et al*., 2004): *A. pallipes*
14	Ticino	Po	-	3	A3 (3)	AY611185 (FRATINI *et al*., 2004): *A. italicus carinthiacus*
20	Lake Caldonazzo	Brenta	Trentino	1	A18 (1)	AY611198 (FRATINI *et al*., 2004): *A. italicus carsicus*
21	Rosandra	Rosandra	Friuli	2	A4 (2)	AY611186 (FRATINI *et al*., 2004): *A. italicus carsicus*
22	Lama	Bidente-Ronco	Emilia Romagna	2	A8 (2)	AY611189 (FRATINI *et al*., 2004): *A. italicus italicus*
23	Farfereta	Arno	Toscana	2	A6 (1); A7 (1)	AY611187; AY611188 (FRATINI *et al*., 2004): *A. italicus italicus*
27	Nera	Tevere	Umbria	1	A14 (1)	AY611193 (FRATINI *et al*., 2004): *A. italicus meridionalis*
31	Samoggia	Reno	Emilia Romagna	/	A19	AF237590 (GRANJDEAN *et al*., 2000): *A. i. italicus*
40	La gace	Clain	France	/	F33	AF237598 (GRANJDEAN *et al*., 2000): *A. pallipes*
43	multiple samplings	Rhone	Switzerland	/	SW1	APA242708 (LARGIADER *et al*., 2000): A. i. carinthiacus
44	multiple samplings	Rhone	Switzerland	/	SW2	APA242709 (LARGIADER *et al*., 2000): *A. i. carinthiacus*

For each site data reported are: water body and its hydrographic drainage, Italian region and/or country, sample size; mitochondrial 16S haplotype. Number 1-10 correspond to the populations sampled for this study; other eleven populations are sequences downloaded from GeneBank with their access number, original source and taxonomic reference; 43-44 are haplotype of more than one water body.



A



B

Figure 2

Molecular phylogeny of *Austropotamobius* spp. inferred by NJ (A) and MP (B) from 301 bp fragment length of mtDNA 16S gene. Bootstrap values are given above the nodes (1,000 replications). *A. torrentium* was used as out-group. The haplotype designations correspond to those reported in Table I.

Figure 2

Phylogénie moléculaire d'*Austropotamobius* spp. établie par NJ (A) et MP (B) à partir de l'analyse de 301 bp du gène codant pour L'ARN ribosomal 16S. Les valeurs de bootstrap sont portées au-dessus des nœuds (1 000 réplications). *A. torrentium* a été utilisé comme out-group. Les haplotypes cités correspondent à ceux reportés dans le tableau I.

Table II

Pairwise mt16S sequence divergence (adjusted for missing data, calculated as *p*-distance = number of substitutions/total number of nucleotide examined) between *Austropotamobius* haplotype estimated by MEGA3 software.

Tableau II

Divergence par paires de séquences mitochondriales 16S (ajustée pour les données manquantes, calculée selon la p-distance = nombre de substitutions/nombre total de nucléotides examinés) entre les haplotypes *Austropotamobius* estimés avec le programme MEGA3.

			A18	A4	M1	M2	A14	M3	SW1	SW2	A3	A19	A6	A7	A8	A5	F33	*A. torr*
Clade A1	A18	*A. italicus carsicus*	-															
	A4	*A. italicus carsicus*	0.006	-														
	M1	*A. italicus carsicus*	0.014	0.009	-													
	M2	*A. italicus carsicus*	0.020	0.014	0.006	-												
Clade A2	A14	*A. italicus meridionalis*	0.023	0.017	0.026	0.032	-											
	M3	*A. italicus meridionalis*	0.029	0.023	0.020	0.026	0.006	-										
Clade A3	SW1	*A. italicus carinthiacus*	0.040	0.034	0.037	0.043	0.029	0.029	-									
	SW2	*A. italicus carinthiacus*	0.043	0.038	0.038	0.043	0.032	0.032	0.003	-								
	A3	*A. italicus carinthiacus*	0.034	0.029	0.032	0.037	0.023	0.023	0.006	0.009	-							
Clade A4	A19	*A. italicus italicus*	0.035	0.032	0.029	0.035	0.032	0.026	0.015	0.018	0.009	-						
	A6	*A. italicus italicus*	0.040	0.040	0.043	0.0449	0.040	0.040	0.023	0.026	0.017	0.009	-					
	A7	*A. italicus italicus*	0.037	0.037	0.040	0.046	0.037	0.037	0.020	0.023	0.014	0.006	0.003	-				
	A8	*A. italicus italicus*	0.034	0.034	0.037	0.043	0.034	0.035	0.017	0.020	0.011	0.003	0.006	0.003	-			
Clade B	A5	*A. pallipes*	0.040	0.040	0.043	0.049	0.037	0.043	0.052	0.052	0.046	0.050	0.055	0.052	0.049	-		
	F33	*A. pallipes*	0.066	0.066	0.063	0.069	0.063	0.063	0.078	0.078	0.072	0.067	0.080	0.078	0.075	0.026	-	
Clade C	AF2375	*A. torrentium*	0.110	0.104	0.098	0.104	0.098	0.095	0.107	0.107	0.101	0.106	0.115	0.112	0.110	0.098	0.118	

Haplotype M1, M2, M3 correspond to the populations sampled for this study; other thirteen populations are sequences downloaded from GeneBank with their access number, original source and taxonomic reference reported in Table I.

may also be the explanation for the presence of populations exhibiting both haplotypes in Amandole and Sanguerone. Humans have had a major impact on organism distribution by transporting animals, especially Crayfish, to many other geographical areas. According to LAURENT and SUSCILLON (1962), REYNOLDS (1998) and LARGIARDER *et al*. (2000), these practices occurred until recently on both the local and larger scales across Europe. The case of Spanish white-clawed crayfish is one of the best examples. Before the application of molecular markers, all authors ascribed a specific or subspecific status to Spanish crayfish, as either *Austropotamobius lusitanicus* or *A. italicus lusitanicus*. However, the molecular data provide a robust phylogeny which does not support this specific or subspecific status (GRANDJEAN *et al*., 2000 and 2002b, TRONTELJ *et al*., 2005). These authors explained the origin of the Spanish stock by a translocation of specimens from Italy. Also the analysis of nine Irish populations of *Austropotamobius pallipes* supported the evidence of the introduction in Ireland from western French specimens (GOUIN *et al.,* 2001).

It was surprising to note that *A. i. italicus* while present in the northern and north-eastern Marches border regions, Emilia Romagna and Tuscany, does not appear in Marches region (FRATINI *et al*., 2005). This could be explained by restricted gene flow between the Adriatic and Mediterranean sides of the Apennine chain.

CONCLUSIONS

Our study confirmed that the application of molecular markers is a prerequisite before considering a management program, in helping taxonomic decisions, especially at specific and intraspecific levels where taxonomic recognition is based on limited numbers of morphological traits. The lack of a precise knowledge of genetic structure could have resulted in establishing populations of allochthonous taxa, and in the genetic contamination of local populations contrary to good conservation management. In terms of conservation genetics, two separated ESU (Evolutionary Significant Units according to Moritz's definition, 1994) occur in Marches. Each should be given conservation priorities and should be managed separately. However, one advantage of such mixed populations is the opportunity to test reproductive isolation between these lineages by the use of nuclear markers.

The next step of this work will be to investigate the genetic variability within populations with more polymorphic mtDNA gene or molecular markers in relation to the hydrographic basins, to improve our knowledge of these populations with the aim to plan conservation projects including restocking and restoration operations in this region. Given the taxonomic unit differences between Emilia Romagna and Marches regions, it would be interesting to verify the probable presence of *A. i. carsicus* in Emilia Romagna, too.

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