Genetic variability in the threatened crayfish Austropotamobius italicus: implications for its management.

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Genetic variability of the threatened crayfish *Austropotamobius italicus* in Tuscany (Italy): implications for its management

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With 4 figures, 4 tables and 1 appendix

Abstract: We studied the genetic variability of the indigenous crayfish *Austropotamobius italicus* in 14 populations inhabiting five basins in Tuscany (Italy). This species is subject to a drastic decline in abundance across its entire range. Using a fragment of the mitochondrial DNA 16S rRNA gene, we identified eight haplotypes, six corresponding to *A. i. italicus* and two to *A. i. meridionalis*; the two clades were found in syntopy in one stream. Eight populations of *A. i. italicus*, analyzed for their microsatellite loci, showed a low intra-population genetic variability and a high inter-population genetic divergence. Populations sampled in the Sieve basin showed no heterozygotes and a high level of inbreeding. The knowledge on the genetic structure of the studied populations, combined with information on their ethology, ecology, and demography, is an essential prerequisite for any action aimed at reintroducing or restocking this threatened species.

Key words: *Austropotamobius italicus*; Decapoda; genetic diversity; mitochondrial DNA; microsatellites; conservation; restocking; threatened species.

Introduction

Understanding the phylogeographic structure of threatened species and assessing their inter- and intra-population genetic variability are prerequisites for any action aimed at optimizing their conservation, such as restocking and reintroduction (Frankel & Soulé 1981, Soulé 1992, O'Brien 1994). Because of their potential to prevent forms of "contamination", genetic studies help maintain the identity and native distribution of the species of conservation concern (Moritz 1994, Avise 2000).

Due to their key role in freshwater communities (e.g. Gherardi et al. 1996, Usio & Townsend 2002, Zhang et al. 2004) and their drastic decline in the last decades, indigenous crayfish species require worldwide urgent action for their conservation (Souty-Grosset et al. 2006a). Paradigmatic is the case of the European species *Austropotamobius pallipes* (Lereboullet, 1858). Originally, it was distributed from the British Isles to Italy and from Portugal to Dalmatia (Holdich & Lowery 1988, Holdich 2002), but since the 1860s' the number and distribution of its populations have been decreased due to several anthropogenic factors, including habitat loss and degradation, overfishing, infectious diseases, and the introduction of non-indigenous species (Gherardi 1999, Gherardi & Holdich 1999, Souty-Grosset et al. 2004, 2006b). Today, the species

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is defined as "vulnerable" by the IUCN (Groombridge 1993) and is included in Annexes II and V of the EU Habitats Directive (92/43/EEC) as a species requiring special conservation measures. Many actions aimed at conserving and restoring *A. pallipes* populations have been undertaken in Europe (Souty-Grosset et al. 2003, 2004, 2006a), but most were hindered by the absence of a well-defined taxonomy and phylogeography (Fratini et al. 2005, Manganelli et al. 2006).

The current taxonomic status of *A. pallipes* (Grandjean et al. 2000, 2006, Grandjean & Souty-Grosset 2000, Fratini et al. 2005) is based on sequences from a fragment of the mitochondrial DNA 16S rRNA gene, a marker frequently used in taxonomy and phylogeography for its maternal inheritance and for the absence of recombination (Avise 1994, Crandall et al. 2000).

Due to the strong genetic (but not morphological) differences found within the *A. pallipes* sp. complex and in agreement with the phylogenetic species concept proposed by Cracraft (1983), two species, *A. pallipes* and *A. italicus*, have been distinguished (Grandjean et al. 2000, Zaccara et al. 2004, Fratini et al. 2005). The two species show a distinct distribution range, *A. pallipes* occurring in Central Europe (France, British Isles, and Northern Italy) and *A. italicus* in Switzerland, Austria, Italy, the Balkan, and Spain (Grandjean et al. 2002a, 2002b). Phylogeographic studies confirmed the co-occurrence in Italy of both *A. pallipes*, confined to the North-Western Italy, and *A. italicus*, distributed across the rest of the Italian peninsula (Fratini et al. 2005). A partial overlap occurs in the Ligurian Apennine (Santucci et al. 1997), but no hybridization event has been recorded (Nascetti et al. 1997, Santucci et al. 1997). A large amount of genetic variation was found in *A. italicus* and four subspecies were proposed, *A. i. carinthiacus*, *A. i. carsicus*, *A. i. italicus*, and *A. i. meridionalis* (Fratini et al. 2005). However, *A. italicus* has not yet been officially recognized as a separate species (Grandjean et al. 2000). This is unfortunate, because any attempt to conserve this species may suffer from the taxonomic ambiguity in the literature (Manganelli et al. 2006). Here, we will use the terminology by Fratini et al. (2005), being aware that this is still provisional (Manganelli et al. 2006).

In the present study, we aimed at characterizing the status and the genetic structure of some populations of *A. italicus* in Tuscany (Italy). The novelty of this study lies in having used two molecular methods, in agreement with the recommendations by Haig (1998) and Souty-Grosset et al. (1999, 2003). In fact, we analyzed (1) mtDNA to sort out the taxonomy and phylogeography of *A. italicus* populations and (2) microsatellites to assess inter- and intra-population genetic variability of *A. i. italicus* populations (Grandjean et al. 1997, Lörtscher et al. 1997, Santucci et al. 1997, Souty-Grosset et al. 1997, Gouin et al. 2002, Paolucci et al. 2004). These two methods combined have the potential to identify Evolutionary Significant Units (ESUs), i.e. "groups of populations that are reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci" (Moritz 1994) and Management Units (MUs), i.e. "populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles" (Moritz 1994).

Material and methods

Sampling

About 200 crayfish were collected by hand from 14 populations inhabiting five hydrographic basins in Tuscany, Italy (Fig. 1). A pereopod was taken from each sampled individual and preserved in absolute ethanol. Appendages are regenerated after successive molts. Crayfish were then immediately released at the collection site.

DNA extraction and amplification

Total genomic DNA was isolated from muscle tissue by multiple extraction methods: Qiagen tissue DNA extraction kit (Qiagen, Valencia, CA, USA) and phenol-chloroform-isoamyl-alcohol method (Kocher et al. 1989), and stored at +4 °C for routine use and at –20 °C for long-term preservation. Polymerase chain reaction (PCR; Mullis et al. 1986) was carried out using a thermal cycler (P E Applied Biosystems GeneAmp model 9700, Perkin-Elmer, USA).

For the mtDNA analysis, we analyzed 70 specimens (five for each population). The selective amplification of a fragment of the mitochondrial DNA 16S rRNA gene (about 550 bp long) was performed by PCR using one set of primers (Fratini et al. 2005). We used primer 1471 (5'-CCTGTTTAN-CAAAACAT-3') and primer 1472 (5'-AGATAGAAACCAAC-CTGG-3') from Crandall & Fitzpatrick (1996), applying the following PCR conditions: 45 cycles for 60 s at 95 °C for denaturation, 60 s at 45 °C for annealing, 60 s at 72 °C for extension, preceded by 3 min of initial denaturation at 95 °C and followed by 5 min of final extension at 72 °C. Successful double-strand PCR products were purified with the Exo-SAP-IT buffer (USB, Cleveland, OH, USA) or a GeneClean II kit (Qbiogene Inc., Carlsbad, CA, USA), and then sequenced using the Big Dye Terminator method on an ABI 377 automated sequencer (PE Applied Biosystem, USA). For most samples, the forward and reverse sequences were obtained.

For the analysis of the microsatellite DNA, we analyzed 15 specimens for each of the 8 *A. i. italicus* populations of study (it is usual to use more samples for analyzing microsatellites, which are much more variable, than for 16S rRNA). We used

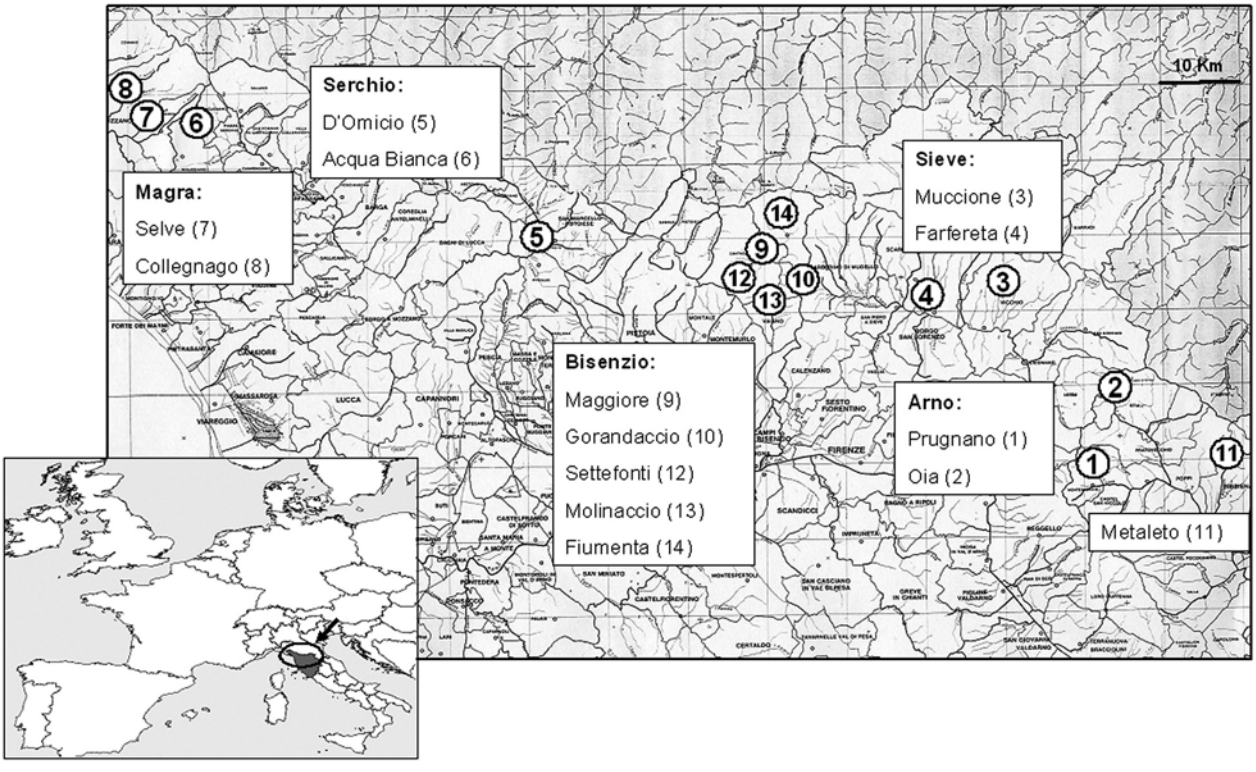

Fig. 1. Map showing the distribution of *Austropotamobius italicus* populations sampled in Tuscany. Basins (Arno, Bisenzio, Magra, Serchio, and Sieve), water bodies, their code (in parentheses), and their location in the map are indicated. Details for each population are given in Table 1.

primers specifically developed for *A. pallipes* by Gouin et al. (2000). Of the four loci, i.e. Ap1, Ap2, Ap3, and Ap6, found to be polymorphic in *A. i. italicus* by Gouin et al. (2000), we obtained results only at the first three microsatellite loci (Ap1, Ap2, and Ap3). For each reaction, one primer of each pair was end-labeled with one fluorescent phosphoramidite (TET, HEX, or 6-FAM) and PCR reactions were performed in a final volume of 12.5 µl, 0.25 U Taq DNA polymerase (Promega, WI, USA), 1.2 mM $MgCl_2$, 60 µM each dNTP, 5 pmol each primer and 15 ng of DNA template. Amplification conditions were 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, specific annealing temperature for 30 s, 72 °C for 45 s and a final extension of 72 °C for 10 min. PCR products were run with the internal size standard GeneScan-500 [TAMRA] (P E Applied Biosystems, USA) on an ABI PRISM 310 automated sequencer (P E Applied Biosystems, USA). Their size was determined using GeneScan Analysis 2.1 (Applied Biosystem, USA).

MtDNA sequence analysis

We analyzed the mtDNA sequences of five individuals for each population. The data matrix of 70 mitochondrial DNA 16S rRNA gene sequences, examined in this study, was supplemented by and compared with other 16 sequences of *Austropotamobius* spp. (Table 1) obtained by Grandjean et al. (2000), Largiadèr et al. (2000), and Fratini et al. (2005). These sequences from GenBank correspond to Italian, French, Swiss, and Slovenian sites (Table 1). Sequences were aligned by eye using the software ESEE Version 3.2 (based on Cabot & Beckenbach 1989). A sequence of *A. torrentium* (AF237599; Grandjean et al. 2000) was included as an outgroup, as was done in previous phylogeographic studies (Grandjean et al. 2000, Largiadèr et al. 2000, Zaccara et al. 2004, 2005, Fratini et al. 2005). Phylogenetic analyses were performed using the neighbour-joining (NJ), the maximum likelihood (ML), and the maximum parsimony (MP) methods. The optimal model of nucleotide evolution for NJ and ML analyses was determined using the AKAIKE INFORMATION CRITERION (AIC) under MODELTEST 4b (Posada & Crandall 1998). For all methods, confidence values of the nodes were evaluated after 1,000 bootstrap re-samplings of the data (Felsenstein 1985), using default options, but with the closest stepwise addition of taxa for MP analyses and the SPR branch swapping option for ML analyses. A median-joining network (MJN) approach (Bandlet et al. 1999) was also conducted to identify the relationships among the white-clawed crayfish haplotypes. This approach has proved to yield the best genealogies among other rooting and network procedures (Cassens et al. 2003); also, it is more convenient to represent relationships among closely related sequences. The median-joining network was estimated using the software NETWORK v. 3.1.1.1 (http://www.flexus-engineering.com).

Microsatellite analysis

The analyses of the microsatellite DNA were conducted in the *A. i. italicus* lineage only, because of its wider distribution in

Table 1. MtDNA analysis: populations of *Austropotamobius* spp. sampled in Tuscany (Italy) (1–14), in other Italian regions (15–24), and outside Italy (25–29). For each population, water body (for codes see Fig. 1), basin (except for population 11, which inhabits Lake of Metaleto), country, sample size, mtDNA 16S rRNA haplotypes (in parentheses, sample size for each haplotype), species/subspecies, GenBank accession number, and source are given. Populations 1–14 have been analyzed in the present study using the molecular technique as indicated (MT: mtDNA; MS: microsatellite). The sequences of populations 15–29 have been downloaded from GeneBank, whereas population 4 has been analyzed in this and in a previous study.

#	Water body	Basin	Sampling sites	*n*	Haplotypes 16 S (*n*)	Species/subspecies	GenBank accession number	Source	Analysis
1	1	Arno	Arezzo	5	I2(3) + I5(2)	*A. i. italicus* + *A. i. meridionalis*	EU308123; EU308126	Present paper	MT
2	2	Arno	Arezzo	5	I5	*A. i. meridionalis*	EU308126	Present paper	MT
3	3	Sieve	Florence	5	A7	*A. i. italicus*	AY611188	Fratini et al. 2005	MT+MS
4	4	Sieve	Florence	5	A6(3) + A7(2)	*A. i. italicus*	AY611187; AY611188	Fratini et al. 2005	MT+MS
5	5	Serchio	Pistoia	5	I1	*A. i. italicus*	EU308122	Present paper	MT+MS
6	6	Serchio	Lucca	5	I4	*A. i. italicus*	EU308125	Present paper	MT+MS
7	7	Magra	Massa	5	I4	*A. i. italicus*	EU308125	Present paper	MT+MS
8	8	Magra	Massa	5	I4	*A. i. italicus*	EU308125	Present paper	MT+MS
9	9	Bisenzio	Prato	5	I1	*A. i. italicus*	EU308122	Present paper	MT+MS
10	10	Bisenzio	Prato	5	I1	*A. i. italicus*	EU308122	Present paper	MT+MS
11	11	–	Arezzo	5	I3	*A. i. italicus*	EU308124	Present paper	MT
12	12	Bisenzio	Prato	5	I5	*A. i. meridionalis*	EU308126	Present paper	MT
13	13	Bisenzio	Prato	5	I5	*A. i. meridionalis*	EU308126	Present paper	MT
14	14	Bisenzio	Prato	5	I5(2) + I6(3)	*A. i. meridionalis*	EU308126; EU308127	Present paper	MT
15	Varaita	Po	Italy	2	A5(2)	*A. pallipes*	AY611201	Fratini et al. 2005	
16	Ticino	Po	Italy	3	A3	*A. i. carinthiacus*	AY611185	Fratini et al. 2005	
17	Lambro	Po	Italy	1	A15	*A. i. carsicus*	AY611195	Fratini et al. 2005	
18	Lambro	Po	Italy	2	A2	*A. i. carinthiacus*	AY611184	Fratini et al. 2005	
19	Lambro	Po	Italy	2	A16(1)	*A. i. carsicus*	AY611196	Fratini et al. 2005	
20	Caldonazzo	Brenta	Italy	1	A18	*A. i. carsicus*	AY611198	Fratini et al. 2005	
21	Rosandra	Rosandra	Italy	2	A4	*A. i. carsicus*	AY611186	Fratini et al. 2005	
22	Duranna	Tevere	Italy	2	A9(1)	*A. i. meridionalis*	AY611190	Fratini et al. 2005	
23	Nera	Tevere	Italy	1	A14	*A. i. meridionalis*	AY611193	Fratini et al. 2005	
24	Cosciale	Crati	Italy	2	A13	*A. i. meridionalis*	AY611192	Fratini et al. 2005	
25	Samoggia	Reno	France	–	A19	*A. i. italicus*	AF237590	Granjdean et al. 2000	
26	La Gace	Clain	France	–	F33	*A. pallipes*	AF237598	Granjdean et al. 2000	
27	Risana	Adriatico	Slovenia	–	A28	*A. i. meridionalis*	AF237593	Granjdean et al. 2000	
28	Several	Rhone	Switzerland	–	SW1	*A. i. carinthiacus*	APA242708	Largiader et al. 2000	
29	Several	Rhone	Switzerland	–	SW2	*A. i. carinthiacus*	APA242709	Largiader et al. 2000	

Tuscany. Fifteen different individuals of the eight *A. i. italicus* populations (populations 3-10) were analyzed. All analyses were performed with the computer package Genetix 4.05.2 (modified after Belkhir et al. 2001). This program was used to calculate allelic frequencies per locus, mean number of alleles per locus (*A*), mean observed heterozygosity (*Ho*), expected heterozygosity (*He*) at each locus within populations, and genetic distances (*D*) between pairs of populations (Nei 1978).

Heterozygosity values were computed as unbiased estimates (Nei 1978). Conformity to Hardy-Weinberg equilibrium at each locus–population combination and globally across loci within populations was analyzed by the GENEPOP computer package version 1.2 (Raymond & Rousset 1995) using the Markov chain method to obtain unbiased estimates of Fisher's exact test through 1,000 iterations (Rousset & Raymond 1995). The software tested also the presence of linkage disequilibrium. Genetic differentiation was assessed using Wright's *F*-statistic (Wright 1951). In particular, F_{st} for pairs of populations and the inbreeding coefficient F_{is} within each population for each locus were estimated by Weir & Cockerham's (1984) method. Genetic differentiation was also calculated as the genetic distances (Nei 1978) between pairs of populations (*D*). The significance of the linkage disequilibrium, F_{st}, and F_{is} was estimated using 1,000 permutations generated from the original genotype matrix (for F_{is} alleles were permuted within samples and for F_{st} genotypes were permuted among samples).

For the analysis of sample homogeneity, following Gouin et al. (2006) we used a Correspondence Analysis (CA) because of both the limited number of loci employed and their relatively low polymorphism. CA was performed on the matrix of allele counts per sample at both the population and the individual level using Genetix 4.05.2 (modified after Belkhir et al. 2001).

When applicable, the significance of the *p*-values was adjusted using the Bonferroni sequential correction for multiple statistical tests (Rice 1989), with an initial value of $0.05/k$ (k being the number of comparisons).

Results

Mitochondrial DNA analysis

A 440 bp fragment of the mitochondrial DNA 16S rRNA gene fragment (primer regions excluded) was analyzed for 70 specimens. We identified eight different haplotypes (Table 1) and 42 variable sites, 27 of which were parsimony informative. Among the 26 sequences described (considering both the populations analyzed here and the additional sequences from GenBank for the comparison), 24 distinct haplotypes were detected (Table 1). Applying the likelihood ratio test procedure, the model of DNA substitution, selected by MODELTEST and applied to the NJ and ML analysis methods, was the HKY85 model (Hasegawa et al. 1985) with unequal substitution rate and with a gamma distribution shape parameter equal to 0.22. The MP method yielded one most parsimonious tree of length 87 (Consistency Index: CI = 0.87, Retention Index: RI = 0.95). Overall all phylogenetic analyses resulted in largely congruent tree topologies (and were well structured with high bootstrap values) and the differences did not affect the general definition of clades or subclades (Fig. 2; see also Fratini et al. 2005).

The obtained phylogenetic inference (due to seven sites among 42 variable sites) seems to support the separation of the haplotypes into two major clades, i.e. clade A, corresponding to the *A. italicus* group, and clade B, corresponding to the *A. pallipes* group (Fig. 2). The average genetic distance (calculated as *p*-distance, i.e. the number of substitutions on the total number of the examined nucleotides, in percentage) is 5.6 % (± 0.13) between the two clades and 0.27 % (± 0.16) within each single clade. The pairwise sequence divergence between haplotypes (Table 2) was calculated as *p*-distance (see above) as shown in the NJ tree in Fig. 2.

Within clade A, four subclades can be distinguished, corresponding to the four *A. italicus* subspecies: *A. i. carinthiacus*, *A. i. carsicus, A. i. italicus,* and *A. i. meridionalis* (Fratini et al. 2005). The sequences of 16S rRNA fragments of the populations examined

Table 2. MtDNA analysis: pairwise sequence divergence (adjusted for missing data, calculated as *p*-distance = number of substitutions/total number of the examined nucleotides, in percentage) between *A. italicus* haplotypes and the outgroup *A. torrentium* (At) obtained from the mtDNA 16S rRNA analysis.

	I1	I2	I3	A7	A6	I4	I5	I6	At
I1	–								
I2	0.009	–							
I3	0.003	0.006	–						
A7	0.015	0.012	0.012	–					
A6	0.018	0.015	0.015	0.003	–				
I4	0.009	0.018	0.012	0.024	0.027	–			
I5	0.030	0.039	0.033	0.045	0.048	0.021	–		
I6	0.033	0.042	0.036	0.048	0.051	0.024	0.003	–	
At	0.110	0.116	0.110	0.122	0.125	0.101	0.096	0.099	–

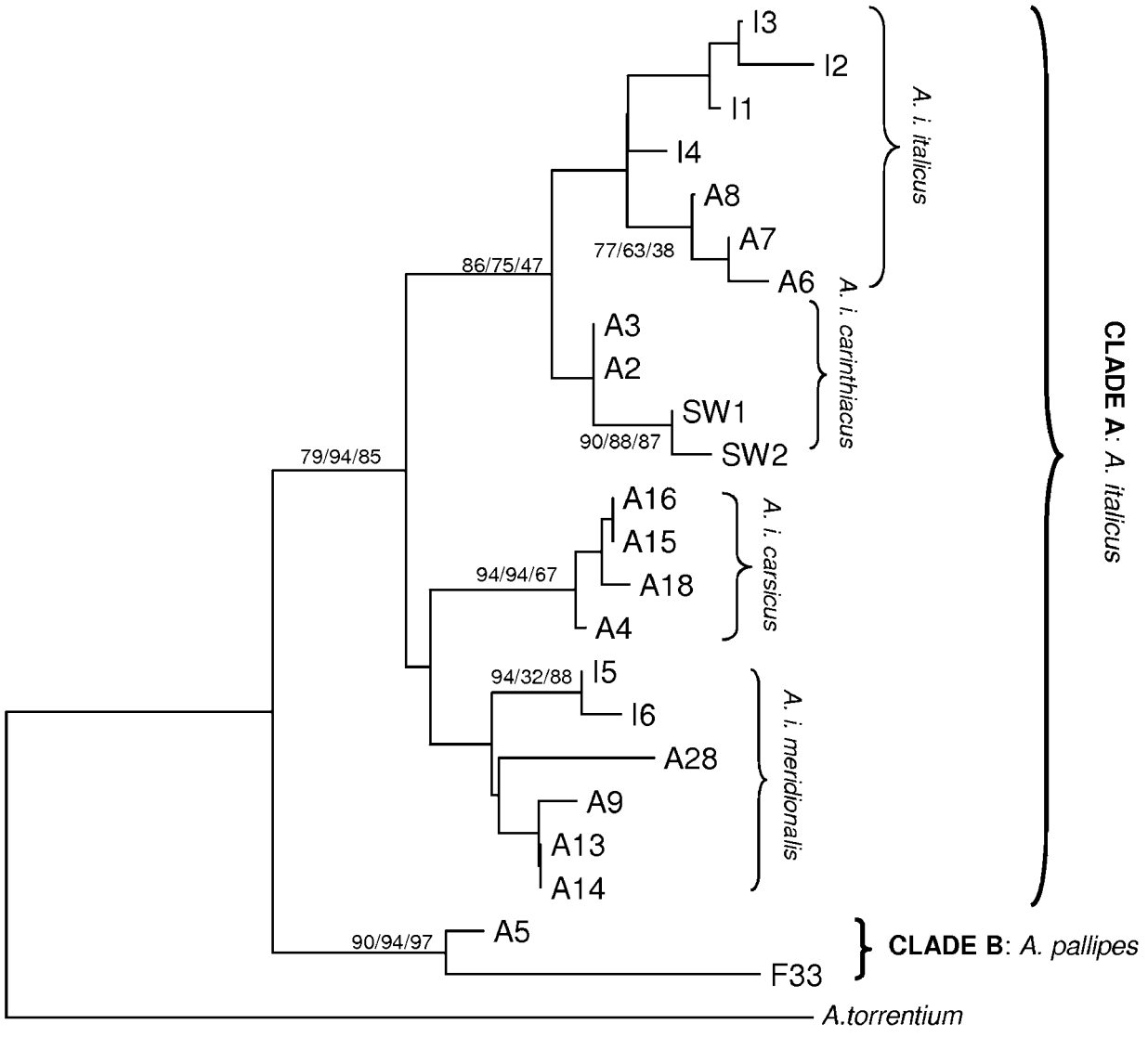

Fig. 2. Neighbour-Joining tree inferred from the analysis of mtDNA 16S rRNA of *Austropotamobius* spp. Clade A corresponds to *Austropotamobius italicus* and clade B to *A. pallipes* lineages; the subclades are *A. i. carinthiacus*, *A. i. carsicus*, *A. i. italicus*, and *A. i. meridionalis*. Bootstrap values are given at nodes (1,000 iterations) for NJ, ML, and MP, respectively (only confidence values higher than 50 % for at least, two of three analyses are shown in the tree). See Table 2 for the haplotypes.

here fell in only one category which corresponds to the *A. italicus* clade, as expected from previous phylogeographic studies. *Austropotamobius i. italicus* is the most frequent haplotype in the 14 analyzed populations (in populations 1 and 3-11), whereas *A. i. meridionalis* was found the least (in populations 1 and 2 and 12–14). In one stream (population 1 in Tables 1 and 2), both *A. i. italicus* and *A. i. meridionalis* were present.

Two sets are evident from the median joining network (Fig. 2). Haplotypes I1–I4 and I5–I6 differed by a minimum of 11 nucleotide substitutions and 3.2 % average sequence divergence (p).

Microsatellite analysis

The three loci, Ap1, Ap2, and Ap3, were polymorphic. Allele frequencies are shown in Appendix 1. The total number of alleles for the eight populations of *A. i. italicus* was 15 and the number of alleles per locus ranged from 2 (locus Ap1) to 7 (locus Ap3) (Appendix 1). Locus Ap6 did not amplify.

The proportion of expected heterozygous individuals per population ranged between 0.00 (population 3, Table 3) and 0.34 (population 10, Table 3) with a mean expected heterozygosity of 0.14. No heterozygote individuals were found in populations 3 and 4, which

Table 3. Microsatellite analysis of *Austropotamobius i. italicus* populations. For each population, stream (see Fig. 1 for codes), basin, and summary statistics for the three analyzed microsatellites are given. *Ho* means the observed heterozygosity, *He* the expected heterozygosity; *A* the number of alleles per locus, and F_{is} the inbreeding coefficient and the deviation from Hardy-Weinberg equilibrium. Significant departures from Hardy–Weinberg equilibrium prior to the sequential Bonferroni corrections are in bold. * and ** mean $p < 0.05$ and $p < 0.01$, respectively.

Population	Basin	*A*	*He*	*Ho*	F_{is}
3	Sieve	1	0	0	**0.99***
4		1	0	0	**0.99***
5	Serchio	1.67	**0.18**	**0.09**	**0.54***
6		1.33	0.04	0.04	–0.04
7	Magra	1.67	0.08	0.09	–0.08
8		2	**0.16**	**0.07**	**0.61***
9	Bisenzio	2.33	0.34	0.24	0.31
10		3	0.33	0.33	0.02

Table 4. Pairwise F_{st} (above the diagonal) and *D*, i.e. Nei's distances (below the diagonal) between *Austropotamobius italicus* populations (see Table 1 for codes). The inhabited basins are indicated. Significant values after Bonferroni sequential correction are in bold.

D \ F_{st}	3 (Sieve)	4 (Sieve)	5 (Serchio)	6 (Serchio)	7 (Magra)	8 (Magra)	9 (Bisenzio)	10 (Bisenzio)
3 (Sieve)		**1**	**0.82**	**0.97**	**0.94**	**0.86**	**0.78**	**0.76**
4 (Sieve)	**0.40**		**0.82**	**0.97**	**0.94**	**0.86**	**0.61**	**0.62**
5 (Serchio)	**0.66**	**0.66**		**0.65**	**0.57**	**0.45**	**0.66**	**0.69**
6 (Serchio)	**1.08**	**1.08**	**0.28**		0.02	0.04	**0.78**	**0.80**
7 (Magra)	**0.99**	0.99	**0.25**	**0.00**		0.02	**0.76**	**0.77**
8 (Magra)	**0.91**	**0.91**	**0.21**	**0.01**	**0.01**		**0.71**	**0.72**
9 (Bisenzio)	**1.35**	**0.40**	**1.28**	**2.30**	**2.20**	**2.04**		0.09
10 (Bisenzio)	**1.04**	**0.41**	**1.66**	**2.89**	**2.58**	**2.42**	**0.09**	

were fixed for a single allele at all loci (both of them are fixed for allele 134 at locus Ap1, for allele 189 at locus Ap3, for allele 196 in population 3, and for allele 194 in population 4 at locus Ap2; Appendix 1).

Fisher's exact test, after sequential Bonferroni correction for multiple tests, gave no significant departure from the Hardy-Weinberg equilibrium in any population, either at individual loci or over all loci (Table 3), with exceptions for populations 5 and 8, and obviously for the monomorphic populations 3 and 4. The first two populations showed significant departure from the Hardy-Weinberg expectations also for F_{is} values which resulted high and significant (population 8: $f = 0.61$; population 5: $f = 0.54$). Private alleles (200, 204, and 206 for Ap2; 215 for Ap3) were also found. Alleles 200 (Ap2) and 215 (Ap3) are also rare with a frequency lower than 0.005 (Hartl & Clark 1997). The highest number of private alleles (204, 206, and 215) was found in population 10, which seemed to be the most differentiated population (with *Ho* = 0.33) along with population 9 (*Ho* = 0.24), both belonging to the Bisenzio basin.

Estimates of *D* (Table 4) reflected the pattern found in F_{st}, (Table 4) with the lowest value of 0.003 between populations 6 and 7 and the highest value of 2.892 between populations 6 and 10. The three couples, i.e. populations 7 and 8 (D = 0.009), 6 and 7 (D = 0.003), and 6 and 8 (D = 0.009), which gave no significant values of F_{st}, showed the lowest but significant *D*. As shown in Table 4, the average genetic distances scored lower between populations inhabiting the same basin compared with populations from different ones. Diver-

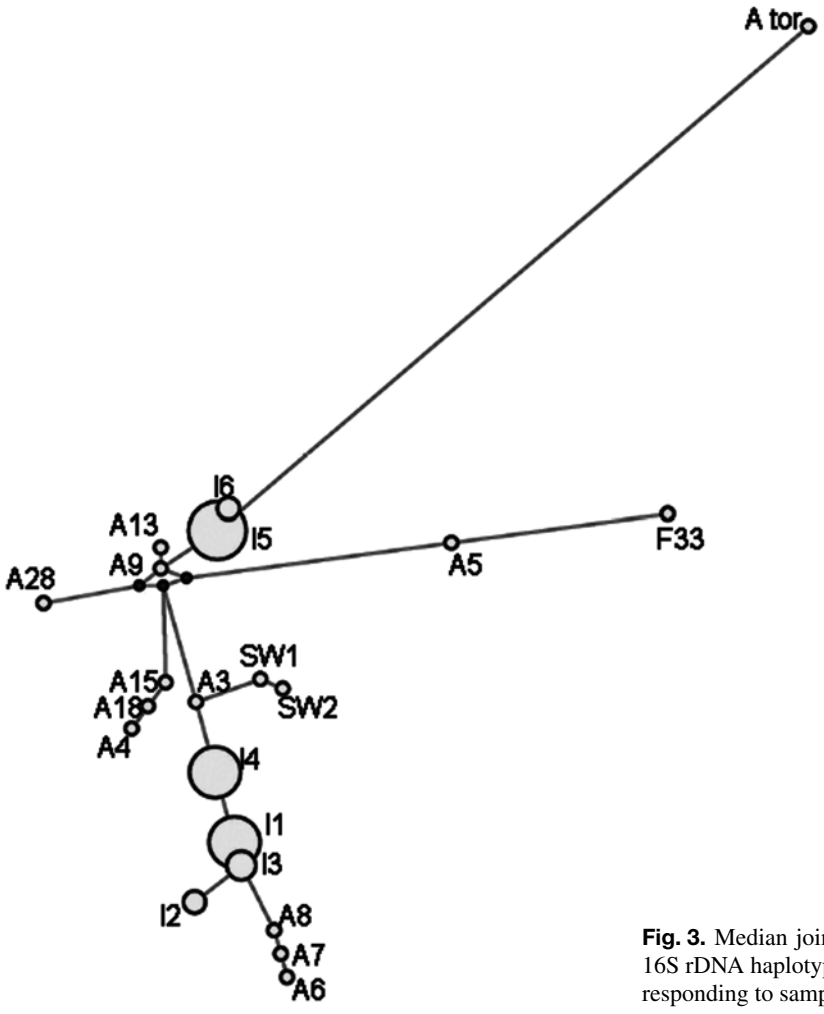

Fig. 3. Median joining network for *Austropotamobius italicus* 16S rDNA haplotypes. Numbered symbols on the network corresponding to samples are listed in Table 1.

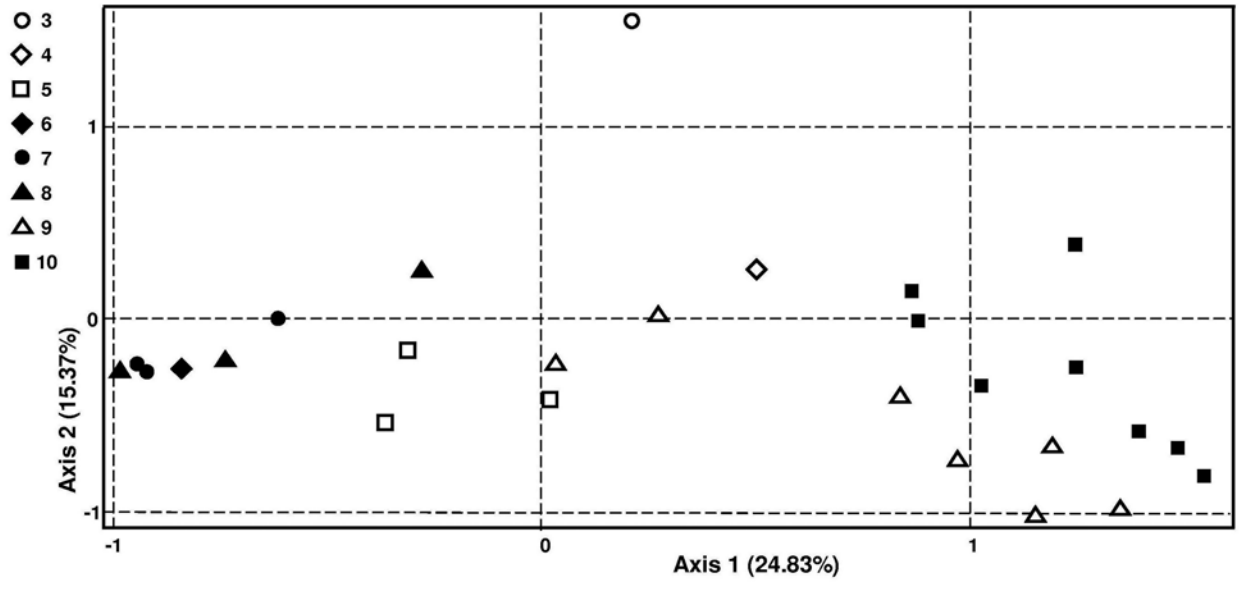

Fig. 4. Correspondence analysis: projection of the population genotypes on the first two axes (see Table 1 for codes). The populations are separated along the first axis with inertia of 24.83 %.

gences are lower for the populations sharing the same mtDNA haplotype.

The pairwise F_{st}-values between populations ranged from 0.09 (between populations 9 and 10) to 0.97 (between populations 3 and 6 and populations 4 and 6) (Table 4). All values (except for the three pairs not in bold in Table 4) were significantly greater than zero. The maximum value was observed between populations 3 and 4.

Microsatellites showed wide inter-population distances but a relatively low intra-population polymorphism. A global and bidimensional view of the genetic intra- and inter-population variability is given by the CA that separates the populations along the first axis, showing inertia of 24.83 % (Fig. 3). Most genotypes of the same population are gathered, indicating low intra-population diversity; each population was separated from the others along the first axis, showing high genetic distances. The populations of different basins (or of different areas) are distant in the plot, suggesting that genetic distances increase with the increase in their geographic distance.

This was confirmed from the other analyses (allelic frequencies per locus, *He*, F_{is}, F_{st}, and *D*), except for the two populations of the Sieve basin (populations 3 and 4). Each of them showed only one genotype, without polymorphism, as apparent in Fig. 3 where populations 3 and 4 are represented by only one point each and are well separated from each other.

Discussion

Our phylogenetic reconstruction based on the mitochondrial DNA 16S rRNA gene analysis confirms that *A. italicus* is the only species occurring in Tuscany (Fratini et al. 2005). Although *A. i. italicus* haplotypes are more frequent, we also found some haplotypes belonging to the *A. i. meridionalis* lineage (haplotypes I6 and I5 in populations 12-14 from the Bisenzio basin, and I5 in populations 1 and 2 from the Arno basin). This is unexpected, because *A. i. meridionalis* is known to have a more southern distribution (Fratini et al. 2005), being found in Abruzzo, Calabria, Campania, and Latium, and reaching the Marches to the north (Cataudella et al. 2006).

The occurrence of this lineage in Tuscany may be explained as an effect of human translocations associated with the traditional use of crayfish as a delicacy. Indeed, translocation of crayfish seems to be a common practice in Western Europe and is extensively documented in the literature (Souty-Grosset et al. 1997, Grandjean et al. 2000, 2001, 2002b, Largiadèr et al. 2000, Gouin et al. 2003, Machino et al. 2004, Holdich et al. 2006). This is also apparent for the artificial Lake of Metaleto (population 11), which harbors a crayfish population with a unique haplotype, probably introduced in the last century by the monks of the nearby Sanctuary of La Verna. Indeed, the suspect of ongoing introductions of crayfish in Tuscany is alarming, since, if uncontrolled, they can compromise the natural genetic pool of the recipient populations, resulting in the loss of their genetic identity due to hybridization with introduced individuals.

Microsatellite analyses have shown a relatively low level of intra-population genetic diversity together with a large differentiation among basins, as suggested by the high values of F_{st} and D (Balloux & Lugon-Moulin 2002). Notwithstanding the low number of microsatellite loci examined here (Koskinen et al. 2004), due to the availability of only four primers for the species under study, our results are congruent with the analysis of the mtDNA. In fact, the identified haplotypes exhibit a well defined geographic distribution, subclades in the phylogenetic tree gathering populations from the same basin or from the same area. These results can be explained from the available information on the demography, ecology, and behavior of the species. The study populations are composed of relatively few individuals, although with a well structured age-class composition (Brusconi et al. 2008). They occupy small areas, frequently isolated by permanent barriers from the main rivers (Renai et al. 2006), and the species in general is known to be conservative in its use of space. Individual crayfish tend to occupy a small home range which is relatively stable with time (Gherardi et al. 1998). Indeed, our finding of the occurrence of private alleles in several populations confirms the absence of any form of gene flow among populations.

The mean number of alleles per locus was low, as expected for populations with a low effective size, where the most common alleles are fixed and the rarest are lost (Hartl & Clark 1997). Except for two streams (populations 9 and 10 in the Bisenzio basin), heterozygosity was relatively low (on average 0.188, excluding populations 3 and 4, which were monomorphic) when compared to studies conducted on the same species (e.g. 0.525 in populations of South Tyrol; Baric et al. 2005).

The two populations of the Sieve basin are not in genetic equilibrium, lack genetic diversity, and show a high rate of inbreeding (high values of F_{is}). A similar phenomenon was found (and explained by restocking)

by Baric et al. (2005) for *A. pallipes* populations in South Tyrol, but for the Sieve basin populations of *A. i. italicus* this may be due to a bottleneck effect. The recorded decline in population size seems to be caused mostly by overexploitation, as shown by Renai et al. (2006) and Brusconi et al. (2008).

Conclusions

Although based on a relatively small sample (but see Roman 2006), our results show the occurrence of eight haplotypes in Tuscany, which is a relatively high number if compared to the total 26 haplotypes found from 36 populations in Italy (Fratini et al. 2005). The eight haplotypes here identified can be grouped into two subspecies, *A. i. italicus* and *A. i. meridionalis*. Data from the microsatellite analyses also denote, within the *A. i. italicus* lineage, genetically independent populations that could be treated as MUs. Most of the populations analyzed for the microsatellite loci are characterized by a low intra-population genetic diversity and a high differentiation across basins (as found for other crayfish species). This knowledge, combined with ethological, ecological, and demographic studies (Gherardi et al. 1998, Renai et al. 2006, Brusconi et al. 2008), is pivotal before undertaking any action of reintroduction or restocking. In fact, this will contribute to identifying stocks with high genetic variability (and therefore more able to resist environmental changes) to which address conservation priorities (Ryder 1986, Soulé & Mills 1992, Primack 2000).

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References

Avise, J. C., 1994: Molecular markers, natural history and evolution. – Chapman and Hall. New York, USA.

– 2000: Phylogeography: the history and formation of species. – Harvard University Press. Cambridge, USA.

Balloux, F. & Lugon-Moulin, N., 2002: The estimation of population differentiation with microsatellite markers. – Molec. Ecol. **11 :** 155–165.

Banarescu, P. M., 1992: Principaux traits de la zoogéographie des eaux douces. – C.R. Soc. Biogéogr. **67:** 133–154.

Bandlet, H. J., Forster, P. & Röhl, A., 1999: Median-joining networks for inferring intraspecific phylogenies. – Molec. Biol. Evolu. **16:** 37–48.

Baric, S., Höllrigl, A., Füreder, L. & Dalla Via, J., 2005: Mitochondrial and microsatellite DNA analyses of *Austropotamobius pallipes* populations in South Tyrol (Italy) and Tyrol (Austria). – Bull. Français Pêche Piscicult. **376-377:**: 599–612.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N., Bonhomme, F., 2001: GENETIX 4.02, logiciel sous Windows TM pour la génétique des populations. Laboratoire génome, populations, interactions, CNRS UMR 5000. – Université de Montpellier II, Montpellier, France.

Brusconi, S., Bertocchi, S., Renai, B., Scalici, M., Souty-Grosset, C. & Gherardi, F, 2008: Stock assessment and habitat requirements in the threatened crayfish *Austropotamobius italicus* in Central Italy: implications for its conservation. – Aquat. Conserv.: Mar. Freshwat. Ecosyst., in press.

Cabot, E. L. & Beckenbach, A. T., 1989: Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. – Computer Appl. Biosci. **5:** 233–234.

Cassens, I., Waerebeek, K., Best, P., Crespo, E., Reyes, J. & Milinkovitch, M., 2003: The phylogeography of dusky dolphins *(Lagenorhynchus obscurus*): a critical examination of network methods and rooting procedure. – Molecul. Ecol. **12:** 1781–1792.

Cataudella, R., Puillandre, N. & Grandjean, F., 2006: Genetic analysis for conservation of *Austropotamobius italicus* populations in Marches Region (Central Italy). – Bull. Français Pêche Piscicult. **380-381:** 991–1000.

Cracraft, J., 1983: Species concepts and speciation analysis. – In: Johnson, R. F. (ed.): Current ornithology. – Plenum Press, New York, pp. 159–187.

Crandall, K. A. & Fitzpatrick, J. F., 1996: Crayfish molecular systematics: using a combination of procedures to estimate phylogeny. – Systematic Biol. **45:** 1–26.

Crandall, K. A., Harris, D. J. & Fetzner, J. W., 2000: The monophyletic origin of freshwater crayfishes estimated from nuclear and mitochondrial DNA sequences. – Proc. Roy. Soc. London, Series B **267:** 1679–1686.

Felsenstein, J., 1985: Confidence limits on phylogenies: an approach using the bootstrap. – Evolution **39:** 783–791.

Frankel, O. H. & Soulé, M. E., 1981: Conservation and evolution. – Cambridge University Press, Cambridge, UK.

Fratini, S., Zaccara, S., Barbaresi, S., Grandjean, F., Souty-Grosset, C., Crosa, G. & Gherardi, F., 2005: Phylogeography of the threatened crayfish (genus *Austropotamobius*) in Italy: implications for its taxonomic and conservation. – Heredity **94:** 108–118.

Gherardi, F., Barbaresi, S. & Villanelli, F., 1998: Movement patterns in the white-clawed crayfish *Austropotamobius pallipes,* in a Tuscan stream. – J. Freshwat. Ecol. **13:** 413–424.

Gherardi, F. & Holdich, D. M. (eds), 1999: Crayfish in Europe as alien species. How to make the best of a bad situation? – A. A. Balkema, Rotterdam, The Netherlands.

Gherardi, F., Villanelli, F. & Dardi, P., 1996: Behavioral ecology of the white-clawed crayfish *Austropotamobius pallipes* in a Tuscan stream: preliminary results. – Freshwat. Crayfish **11:** 182–193.

Gouin, N., Grandjean, F., Pain, S., Souty-Grosset, C. & Reynolds, J., 2003: Origin and colonization history of the white-clawed crayfish, *Austropotamobius pallipes*, in Ireland. – Heredity **91:** 70–77.

Gouin, N., Grandjean, F. & Souty-Grosset, C., 2000: Characterization of microsatellite loci in the endangered freshwater crayfish *Austropotamobius pallipes* (Astacidae) and their potential use in other decapods. – Molecul. Ecol. **9:** 629–644.

Gouin, N., Grandjean, F. & Souty-Grosset, C., 2006: Population genetic structure of the endangered crayfish *Austropotamobius pallipes* in France based on microsatellite variation: biogeographical inferences and conservation implications. – Freshwat. Biol. **51:** 1369–1387.

Gouin, N., Souty-Grosset, C., Ropiquet, A. & Grandjean, F., 2002: High dispersal ability of *Austropotamobius pallipes* revealed by microsatellite markers in a French brook. – Bull. Français Pêche Piscicult. **367:** 681–689.

Grandjean, F., Bouchon, D. & Souty-Grosset, C., 2002a: Systematic of the European endangered crayfish species *Austropotamobius pallipes* (Decapoda: Astacidae) with a re-examination of the status of *Austropotamobius berndhauseri*. – J. Crustacean Biol. **22:** 677–681.

Grandjean, F., Frelon-Raimond, M. & Souty-Grosset, C., 2002b: Compilation of molecular data for the phylogeny of the genus *Austropotamobius*: one species or several? – Bull. Français Pêche Piscicult. **367:** 671–680.

Grandjean, F., Gouin, N., Keith, P., Noel, P., Persat, H., Reynolds, J., Schulz, H., Smietana, P. & Souty-Grosset, C., 2006: Systematics and phylogeny of freshwater crayfish, with particular reference to historical biogeography of Europe. – In: Souty-Grosset, C., Holdich, D., Noël, P., Reynolds, J. & Haffner, P. (eds): Atlas of crayfish in Europe. – Museum National d'Histoire Naturelle, Paris, France, pp. 11–23.

Grandjean, F., Gouin, N., Souty-Grosset, C., Dieguez-Uribeondo, J., 2001: Drastic bottleneck in the endangered crayfish species *Austropotamobius pallipes* in Spain and implication for its colonization history. – Heredity **86:** 1–8.

Grandjean, F., Harris, D. J., Souty-Grosset, C. & Crandall, K. A., 2000: Systematics of the European endangered crayfish species *Austropotamobius pallipes* (Decapoda: Astacidae). – J. Crustacean Biol. **20:** 522–529.

Grandjean, F. & Souty-Grosset, C., 2000: Mitochondrial DNA variation and population genetic structure of the white-clawed crayfish, *Austropotamobius pallipes pallipes*. – Conservation Genetics **1:** 309–319.

Grandjean, F., Souty-Grosset, C., Raimond, R. & Holdich, D. M., 1997: Geographical variation of mitochondrial DNA between European populations of the white-clawed crayfish *Austropotamobius pallipes*. – Freshwat. Biol. **371:** 493–501.

Groombridge, B., 1993: IUCN red list of threatened animals. – IUCN, Gland, Switzerland and Cambridge, UK.

Haig, S. M., 1998: Molecular contributions to conservation. – Ecology **79:** 413–425.

Hartl, D. L, & Clark, A. J., 1997: Principles of population genetics. – Sinauer Associates, Inc. Sunderland, USA.

Hasegawa, M., Kishino, K. & Yano, T., 1985: Dating the human–ape splitting by a molecular clock of mitochondrial DNA. – J. Molecular Evol. **22:** 160–174.

Holdich, D. M., 2002: Present distribution of crayfish in Europe and some adjoining countries. – Bull. Français Pêche Piscicult. **367:** 611–650.

Holdich, D. M., Haffner, P., Noël, P., Carral, J., Füreder, L., Gherardi, F., Machino, Y., Madec, J., Pöckl, M., Smietana, P., Taugbol, T. & Vigneux, E., 2006: Systematics and phylogeny of freshwater crayfish, with particular reference to historical biogeography of Europe. – In: Souty-Grosset, C., Holdich, D., Noël, P., Reynolds, J. & Haffner, P. (eds): Atlas of crayfish in Europe. – Museum National d'Histoire Naturelle, Paris, France, pp. 50–129.

Holdich, D. M. & Lowery, R. S., 1988: Freshwater crayfish: Biology, management and exploitation. – Croom Helm. London, UK, and Sydney, Australia.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Paabo, S. & Villablancha, F. X., 1989: Dynamics of mtDNA evolution in animals: amplification and sequencing with conserved primers. – Proc. Nation. Acad. Sci. **86:** 6196–6200.

Koskinen, M. T., Hirvonen, H., Landry, P.-A. & Primmer, C. R., 2004. The benefits of increasing the number of microsatellites utilized in genetic population studies: an empirical perspective. – Hereditas **141**: 61–67.

Largiadèr, C. R., Herger, M., Lörtscher, M. & Scholl, A., 2000: Assessment of natural and artificial propagation of the white-clawed crayfish (*Austropotamobius pallipes* species complex) in the Alpine region with nuclear and mitochondrial markers. – Molecul. Ecol. **9:** 25–37.

Lörtscher, M., Stucki, T. P., Clalüna, M. & Scholl, A., 1997: Phylogeographic structure of *Austropotamobius pallipes* populations in Switzerland. – Bull. Français Pêche Piscicult. **347:** 649–661.

Machino, Y., Füreder, L., Laurent, P. J. & Petutschnig, J., 2004: Introduction of the white-clawed crayfish *Austropotamobius pallipes* in Europe. – Ber. naturwiss-medizin. Vereins Innsbruck **91:** 187–212.

Manganelli, G, Favilli, L, Fiorentino, V., 2006: Taxonomy and nomenclature of Italian white-clawed crayfish. – Crustaceana **79:** 633–640.

Moritz, C., 1994: Defining 'Evolutionary Significant Units' for conservation. – Trends Ecol. Evolut. **9:** 373–375.

Mullis, K. B., Faloona, F., Scharf, S., Saiki, R. K., Horn, G. & Erlich, H., 1986: Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbour Symposium. – Quantitative Biol. **51:** 263–273.

Nascetti, G., Andreani, P., Santucci, F., Iaconelli, M. & Bullini, L., 1997: Struttura genetica di popolazioni italiane di gambero di fiume (*Austropotamobius pallipes*) e strategie per la sua conservazione. – Atti della Società Italiana di Ecologia **18:** 205–208.

Nei, M., 1978: Estimation of average heterozigosity and genetic distance from a small number of individuals. – Genetics **89:** 583–590.

O'Brien, S. J., 1994: Genetic and phylogenetic analyses of endangered species. – Annu. Rev. Genetics **28:** 467–489.

Paolucci, M., Liberato, C., Di Cristo, C. & Di Cosmo, A., 2004: Freshwater crayfish populations in the district of Benevento (Campania Region, Italy): distribution and analysis of genetic structure. – Freshwat. Crayfish **14:** 121–128.

Posada, D. & Crandall, K. A., 1998: Testing the model of DNA substitution. – Bioinformatics **14:** 817–818.

Primack, R. B., 2000: Conservation at the population and species level. – In: Primack, R. B. (ed.): *A* Primer of Conservation Biology, 2nd ed. – Sinauer Associates: Sunderland, USA, pp. 121−182.

Raymond, M. & Rousset, F., 1995: GENEPOP (Version 1.2): Population genetics software for exact tests and ecumenicism. – J. Heredity **86:** 248–249.

Renai, B., Bertocchi, S., Brusconi, S., Gherardi, F., Grandjean, F., Lebboroni, M., Parinet, B., Souty-Grosset, C. & Trouilhé, M. C., 2006: Ecological characterization of streams in Tuscany (Italy) for the management of the threatened crayfish *Austropotamobius italicus*. – Bull. Français Pêche Piscicult. **380-381:** 1095–1114.

Rice, W. R., 1989: Analysing tables of statistical tests. – Evolution **43**: 223–225.

Roman, J., 2006: Diluting the founder effect: cryptic invasions expand a marine invader's range. – Proc. Roy. Soc. London B **273:** 2453–2459.

Rousset, F. & Raymond, M., 1995: Testing heterozygote excess and deficiency. – Genetics **140:** 1413–1419.

Ryder, O. A., 1986: Species conservation and systematic: the dilemma of subspecies. – Trends Ecol. Evolut. **1:** 9–10.

Santucci, F., Iaconelli, M., Andreani, P., Cianchi, R., Nascetti, G. & Bullini, L., 1997: Allozyme diversity of European freshwater crayfish of the genus *Austropotamobius*. – Bull. Français Pêche Piscicult. **347:** 663–676.

Soulé, M. E., 1992: Conservation biology. – In: Soulé, M. E. (ed.): The science of scarcity and diversity Sinauer Associates, Inc. Publishers, Sunderland, USA.

Soulé, M. E. & Mills, L. S., 1992: Conservation genetics and conservation biology: a troubled marriage. – In: Saundlund, O. T., Hindar, K. & Brown, A. H. D. (eds): Conservation of biodiversity for sustainable development. – Scandinavian University Press, Oslo, Norway, pp. 55–69.

Souty-Grosset, C., Carral, J., Edsman, L., Füreder, L., Gherardi, F., Grandjean, F., Haffner, P., Holdich, D., Madev, J., Mannonen, A., Noël, P., Reynolds, J., Schulz, R., Smietana, P. & Taugbøl, T., 2006a: Conservation of European crayfish, a challenge involving everybody, from the citizen to stakeholders, scientists and decision makers. – In: Souty-Grosset, C., Holdich, D., Noël, P., Reynolds, J. & Haffner, P. (eds): Atlas of crayfish in Europe. – Museum National d'Histoire Naturelle: Paris, France, pp. 159–162.

Souty-Grosset, C., Grandjean, F. & Gouin, N., 1999: Molecular genetic contributions to conservation biology of the European native crayfish *Austropotamobius pallipes*. – Freshwat. Crayfish **12:** 371–386.

– – – 2003: Keynote involvement of genetics in knowledge, stock management and conservation of *Austropotamobius pallipes* in Europe. – Bull. Français Pêche Piscicult. **370-371:** 165–179.

– – – 2004: Conservation and management of native crayfish populations. – Freshwat. Crayfish **14:** 1–14.

Souty-Grosset, C., Grandjean, F., Raimond, R., Frelon, M., Debenest, C. & Bramard, M., 1997: Conservation genetics of the white-clawed crayfish *Austropotamobius pallipes*: usefulness of the mitochondrial DNA marker. – Bull. Français Pêche Piscicult. **347:** 677–692.

Souty-Grosset, C., Holdich, D., Noël, P., Reynolds, J. & Haffner, P. (eds), 2006b: Atlas of crayfish in Europe – Museum National d'Histoire Naturelle: Paris, France, 187 pp.

Usio, N. & Townsend, C. R., 2002: Functional significance of crayfish in stream food webs: roles of omnivory, substrate heterogeneity and sex. – Oikos **98:** 512–522.

Weir, B. S. & Cockerham, C. C., 1984: Estimating F-statistics for the analysis of population structure. – Evolution **38:** 1358–1370.

Wright, S., 1951: The genetical structure of populations. – Ann. Eugenics **15:** 323–354.

Zaccara, S., Stefani, F. & Crosa, G., 2005: Diversity of mitochondrial DNA of the endangered white-clawed crayfish (*Austropotamobius pallipes*) in the Po River catchment. – Freshwat. Biol. **50:** 1262–1272.

Zaccara, S., Stefani, F., Galli, P., Nardi, P. A. & Crosa, G., 2004: Taxonomic implications in conservation management of white-clawed crayfish (*Austropotamobius pallipes*) (Decapoda, Astacidae) in Northern Italy. – Biol. Conservation **120:** 1–10.

Zhang, Y., Richardson, J. S. & Negishi, J. N., 2004: Detritus processing, ecosystem engineering and benthic diversity: a test of predator–omnivore interference. – J. Animal Ecol. **73:** 756–766.

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Appendix 1. Allele frequencies for each microsatellite locus in each sampled population. See Table 1 for codes.

	Population							
Locus	3	4	5	6	7	8	9	10
Ap1								
130	–	–	–	–	–	–	0.77	0.87
134	1	1	1	1	1	1	0.23	0.13
Ap2								
194	–	1	–	–	–	–	1	0.83
196	1	–	–	–	–	–	–	0.07
200	–	–	–	–	–	0.03	–	–
202	–	–	1	1	1	0.97	–	–
204	–	–	–	–	–	–	–	0.07
206	–	–	–	–	–	–	–	0.03
Ap3								
171	–	–	–	0.93	0.86	0.73	–	–
187	–	–	–	–	0.07	–	–	–
189	1	1	0.4	–	0.07	0.1	0.4	0.67
191	–	–	–	0.07	–	0.17	0.1	–
215	–	–	–	–	–	–	–	0.03
217	–	–	0.07	–	–	–	0.1	0.3
219	–	–	0.53	–	–	–	0.4	–