

SYNTOPY OF *A. PALLIPES* AND *A. ITALICUS*: GENETIC AND MORPHOMETRICAL INVESTIGATIONS

D. GHIA (1), P.A. NARDI (1), A. NEGRI (2), F. BERNINI (1), A. BONARDI (1),
G. FEA (1), M. SPAIRANI (1)

(1) Dipartimento di Biologia Animale, Università degli Studi di Pavia, P.zza Botta 9, 27100 Pavia, Italy.
acquint@unipv.it

(2) Dipartimento di Scienze dell'Ambiente e della Vita, Università del Piemonte Orientale, Via Bellini 25/G, 15100 Alessandria, Italy.

Reçu le 28 juin 2005
Accepté le 12 janvier 2006

Received June 28, 2005
Accepted January 12, 2006

ABSTRACT

Genetic studies on the populations of *Austropotamobius pallipes* complex from Alessandria province (N-Italy), conducted on the basis of variations recorded in the sequences of mitochondrial DNA in the gene 16S RNA, have allowed to single out an overlapping zone of the distribution areas of the two taxa *pallipes* and *italicus*. In the crayfishes from a creek where both species have been ascertained to be present, the authors have applied the same molecular marker in order to investigate the distribution of the two taxa, stressing the syntopy of *A. pallipes* and *A. italicus*. Multivariate statistical techniques on 21 morphological parameters have been used in order to single out possible distinctive features of 31 crayfishes collected in this site, and of 225 crayfishes obtained from four sites in neighbouring areas. The results confirm the presence of a high morphological variability, not only at interspecific, but also at intraspecific level, which does not allow to distinguish the two species on the basis of the features measured in the present study.

Key-words: *Austropotamobius pallipes*, *Austropotamobius italicus*, mitochondrial DNA, morphometry.

SYNTOPIE DE *A. PALLIPES* ET *A. ITALICUS*: INVESTIGATIONS GÉNÉTIQUES ET MORPHOMETRIQUES

RÉSUMÉ

Les études génétiques menées sur les populations du complexe d'*Austropotamobius pallipes* dans la province d'Alessandria (N-Italie) ont été faites sur la base des variations mesurées dans les séquences de l'ADN mitochondrial dans le gène 16S ARN. Elles ont permis de mettre en évidence une zone de superposition des distributions des deux taxa *pallipes* et *italicus*. On a utilisé le même marqueur moléculaire sur les deux espèces d'écrevisses provenant du même petit ruisseau, pour étudier leurs distributions et mettre en évidence la syntopie de *A. pallipes* et *A. italicus*. Des analyses statistiques multivariées ont été appliquées sur 21 paramètres morphologiques pour mettre en relief de possibles caractéristiques distinctives sur 31 écrevisses capturées dans ce ruisseau et

sur 225 écrevisses prises dans quatre sites des zones voisines. Les résultats confirment la présence d'une haute variabilité morphologique, non seulement interspécifique mais également intraspécifique, qui ne permet pas de distinguer les deux espèces sur la base des caractéristiques mesurées dans cette étude.

Mots-clés : *Austropotamobius pallipes*, *Austropotamobius italicus*, ADN mitochondrial, morphométrie.

INTRODUCTION

The current taxonomic position of white-clawed crayfish, based on morphological (BOTT, 1950, 1972; KARAMAN, 1962; BRODSKY, 1983) and allozymatic studies (NASCETTI *et al.*, 1997; SANTUCCI *et al.*, 1997) and confirmed by 16S mtDNA (GRANDJEAN *et al.*, 2000; GRANDJEAN, BOUCHON and SOUTY-GROSSET, 2002), shows *Austropotamobius pallipes* as a species complex, with two genetically well recognizable species, *A. pallipes* and *A. italicus*. On the contrary, the taxonomy based on morphological criteria (BOTT, 1950, 1972; KARAMAN, 1962; LAURENT and SUSCILLON, 1962) seems difficult and moot because morphological characters are often changeable both among individuals of the same population and among different populations within species (ALBRECHT, 1982; ATTARD and VIANET, 1985; GRANDJEAN *et al.*, 1997a; LÖRTSCHER *et al.*, 1997). Recently, genetic studies have revealed the presence of the two species in NW Italy and an overlapping area in the province of Alessandria, where *pallipes* and *italicus* appear to be sympatric in at least two of the brooks examined (NEGRI *et al.*, 2003; ZACCARA *et al.*, 2004).

Our research focussed on the genetic investigation on the crayfish populations from the province of Alessandria and, in particular, on the syntopic populations living in the Lagoscuro Brook, in order to verify their actual syntopy, to define their distribution along the watercourse, and to be able to discriminate the two species in the field on the basis of some morphometric features.

MATERIALS AND METHODS

In the province of Alessandria (NW Italy) a total of 145 specimens of *A. pallipes* complex were collected from 25 Po River affluents. In order to verify the syntopy, 47 of these crayfishes were sampled in three distinct stretches (each one about 100 m long) picked out in the terminal part (about 500 m long) of the Lagoscuro Brook: between 14 and 18 crayfishes of both sexes were collected by hand in night time for each stretch. For the subsequent morphometric analyses, the populations from the Lemme Torrent and Angiassi Brook have been genetically checked in the present study, analysing 16 specimens for each of the two watercourses; the species identity of the populations from Schizzola Torrent and Reponte Brook was already known in the literature (ZACCARA *et al.*, 2004). A claw or a pereopod was taken from each individual and was immediately put in a vial containing absolute ethanol and stored at – 80°C for DNA isolation.

Genetic analyses

DNA isolation and amplification

Total genomic DNA was isolated from muscle tissue using NucleoSpin Tissue kit (Macherey-Nagel). The DNA were eluted in a buffer supplied by the kit and stored at – 20°C. A 520 bp fragment of the mt16S DNA gene was amplified using primers 16Sar-L (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16Sbr-H (5'-CCG GTC TGA ACT CAG ATC ACG T-3') (PALUMBI *et al.*, 1991). Polymerase chain reaction was carried out using a programmable thermal cycler (PCR Express-Hyaid). PCR reaction was performed in a final volume of

25 µl: 1 µl DNA, 0.5 µl of each primer (25 µM), 0.5 µl of DNTP mixture (0.2 µM), 1 U of Red Taq (Sigma-Aldrich), 2.5 of 10X buffer and 20 µL of MQ water. Selective amplification was carried out by PCR cycles: 30 cycles, 2 min at 95°C for one cycle, 95°C/30 s, 48°C/30 s, 72°C/30 s denaturing, annealing and extension temperatures. After the last cycle a final extension was carried out at 72°C for 5 min. PCR products were purified, using enzymatic procedure (EXO-SAP-IT buffer, USB®). These products were sequenced using ABI PRISM 3730XL DNA Sequencer (Applied Biosystem).

Sequence analysis

The resulting sequences were aligned using Clustal X 1.81 (THOMPSON *et al.*, 1997). The data matrix included the 145 sequences investigated in this work and 13 sequences of *A. pallipes* and *A. italicus* obtained by FRATINI *et al.* (2005) and ZACCARA

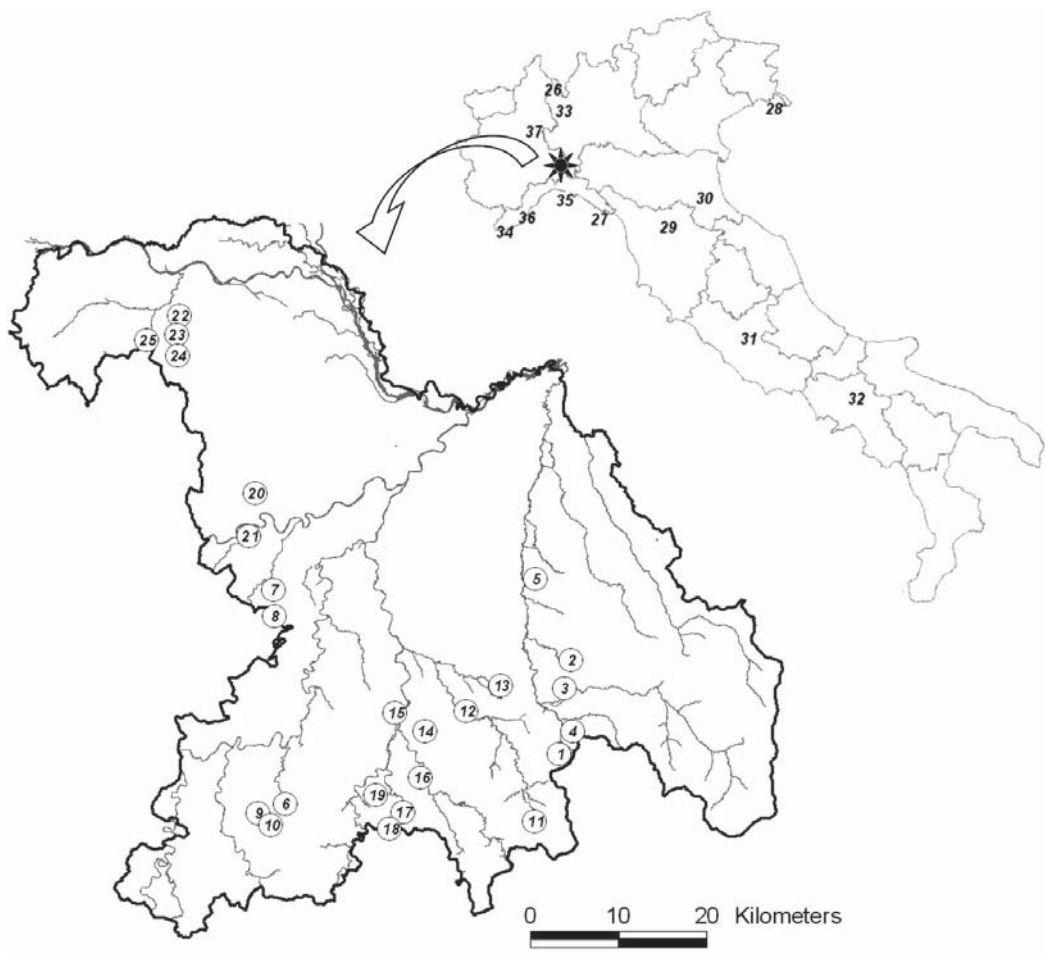


Figure 1

Sampling sites of white-clawed crayfish in the province of Alessandria (analysed in this study, on the left) and in Italy (downloaded from GenBank, on the right).(Codes as in table I).

Figure 1

Localisation géographique des sites d'échantillonnage de l'écrevisse à pattes blanches dans la province d'Alessandria (zone d'étude, à gauche) et en Italie (téléchargés de GenBank, à droite). (Même numérotation que dans le Tableau I).

Table I

1-25: Alessandria white-clawed crayfish populations analysed in this study; 26-37: sequences downloaded from GenBank (Italian haplotypes). (*) Haplotypes from more than one watercourse.

Tableau I

1-25 : populations des écrevisses à pattes blanches de la province d'Alessandria analysées dans cette étude ; 26-37 : séquences téléchargées de GenBank (haplotypes italiens). (*) Haplotypes provenant de plusieurs rivières.

Code	Hydrographic drainage	Water body	N°	HAPLOTYPE	Taxonomic reference GenBank accession number and original source
1	Scrvia	Mulino	3	SB; LEM1B; LG2	<i>A. i. carinthetaicus</i> ; <i>A. pallipes</i>
2	Scrvia	Angiassi	16	SB	<i>A. i. carinthetaicus</i>
3	Scrvia	Lagoscuoro	47	SB; LG20; LG15; LG2; LG34; LG19	<i>A. i. carinthetaicus</i> ; <i>A. pallipes</i>
4	Scrvia	S. Bartolomeo	3	LG15; LEM1B	<i>A. pallipes</i>
5	Scrvia	Vaccaruzza	3	SB	<i>A. i. carinthetaicus</i>
6	Bormida	Visone	7	GAMB	<i>A. i. meridionalis</i>
7	Bormida	Ghisona	3	SB	<i>A. i. carinthetaicus</i>
8	Bormida	Gattini	2	SB	<i>A. i. carinthetaicus</i>
9	Bormida	Lavandera	2	LAV2B; GAMB	<i>A. i. meridionalis</i>
10	Bormida	Gambarello	2	GAMB	<i>A. i. meridionalis</i>
11	Orba	Lemme	16	LEM1B	<i>A. pallipes</i>
12	Orba	Lagoraro	4	SB	<i>A. i. carinthetaicus</i>
13	Orba	Pareto	3	SB	<i>A. i. carinthetaicus</i>
14	Orba	Arbara	4	SB	<i>A. i. carinthetaicus</i>
15	Orba	Maggiore	4	SB	<i>A. i. carinthetaicus</i>
16	Orba	Piota	1	SB	<i>A. i. carinthetaicus</i>
17	Orba	Coda	3	SB	<i>A. i. carinthetaicus</i>
18	Orba	Ceci	2	SB	<i>A. i. carinthetaicus</i>
19	Orba	Requagliolo	2	SB	<i>A. i. carinthetaicus</i>
20	Tanaro	Sabbiona	5	SB	<i>A. i. carinthetaicus</i>
21	Tanaro	Redabue	2	SB	<i>A. i. carinthetaicus</i>
22	Po	Treville	4	SB	<i>A. i. carinthetaicus</i>
23	Po	Monrabbioso	3	SB	<i>A. i. carinthetaicus</i>
24	Po	Sturella	3	SB	<i>A. i. carinthetaicus</i>
25	Po	Colobrio	1	SB	<i>A. i. carinthetaicus</i>
26*	Po	Lambro	/	A2	<i>A. i. carinthetaicus</i> AY611184 (Fratini et al., 2005)
27*	Magra	Gottero	/	A3	<i>A. i. carinthetaicus</i> AY611185 (Fratini et al., 2005)
28	Rosandra	Rosandra	/	A4	<i>A. i. carsicus</i> AY611186 (Fratini et al., 2005)
29	Arno	Farfereta	/	A6	<i>A. i. italicus</i> AY611187 (Fratini et al., 2005)
29	Arno	Farfereta	/	A7	<i>A. i. italicus</i> AY611188 (Fratini et al., 2005)
30*	Bidente-Ronco	Lama	/	A8	<i>A. i. italicus</i> AY611189 (Fratini et al., 2005)
31	Tevere	Duranna	/	A9	<i>A. i. meridionalis</i> AY611190 (Fratini et al., 2005)
32*	Sele	S. Antuono	/	A14	<i>A. i. meridionalis</i> AY611193 (Fratini et al., 2005)
33*	Po	Lambro	/	A17	<i>A. i. carsicus</i> AY611197 (Fratini et al., 2005)
34	Argentina	Oxentina	/	A21	<i>A. pallipes</i> AF237597 (Grandjean et al., 2000)
35	Bisagno	Arvigo	/	A22	<i>A. pallipes</i> AY611202 (Fratini et al., 2005)
36	Po	Montenotte	/	A24	<i>A. pallipes</i> AY611204 (Fratini et al., 2005)
37*	Po	Sesia	/	SS2	<i>A. pallipes</i> AY521287 (Zaccara et al., 2004)

et al. (2004) and deposited in GenBank used for a systematic scheme of the sampled individuals (Figure 1 and Table I).

The tree reconstructions were performed using the neighbour-joining (NJ) and maximum parsimony (MP) analysis implemented in PAUP (SWOFFORD, 1998). MP analyses were performed using a heuristic search, based on branch swapping with tree bisection reconnection for identifying parsimonious-trees, summarized in a strict consensus-tree. Insertion and deletion gaps were treated as missing data. A sequence of *A. torrentium* downloaded from GenBank was used as outgroup in order to uniform the phylogenetic interpretation (ZACCARA *et al.*, 2004; FRATINI *et al.*, 2005). Support for the internodes in tree was assessed by bootstrap percentages (BP; FELSENSTEIN, 1985) after 1000 resampling steps, rejecting values lower than 50.

Morphometric analyses

From 31 crayfishes collected in the Lagoscuro Brook, from 99 specimens of *A. pallipes* collected in the Lemme Torrent and from a total of 126 specimens of *A. italicus* collected in three other watercourses (Angiassi Brook in the province of Alessandria; Schizzola Torrent and Reponte Brook in Oltrepò pavese – Lombardy), the same researcher recorded 21 morphometric parameters (Table II), according to several authors

Tableau II
Morphometric parameters.

Tableau II
Paramètres morphométriques.

Code	Description
A	Total Length
B	Cephalotorax Length (from the tip of the rostrum to the posterior median edge of the cephalotorax)
C	Transversal Cephalotorax Length (from the posterior orbital edge to the posterior median edge of the cephalotorax)
D	Maximum Cephalotorax Length
E	Claw Length
G	Dactylopodite Length
I	Claw Width
L	Tip Rostrum Length
M	Back Rostrum Length
N	Maximum Cephalotorax Width
O	Width between Eyes
P	Rostrum Width
Q	Cervical Groove Width
R	Branchio-cardiac Groove Width
S	Cephalotorax Window Width
T	II Abdominal Segment Width
U	III Abdominal Segment Width
V	Telson Width
X	Branchio-cardiac Groove Length
Y	Abdominal Length
Z	Telson Length

(ARRIGNON, 1996; GRANDJEAN *et al.*, 1997b; SINT, DALLA VIA and FÜREDER, 2004) to the nearest 0.01 mm using a digital caliper. After measurements, most crayfishes were returned to their sampling site, except few voucher specimens preserved in 70% ethanol, for future morphological studies. For the statistical analyses we excluded total length (A), regenerating claws and individuals having carapace length < 15 mm. All measurements considered were log-transformed for statistical analyses.

The sexes were analyzed separately, as an obvious sexual dimorphism is widely known for *Austropotamobius* spp. (MORI, MANDICH and ISOLA, 1991; GRANDJEAN *et al.*, 1997b; STREISSL and HÖDL, 2002).

Since linear measurements of morphological characters are strongly correlated with body size, we used as size-adjusted shape variables the set of residuals from regressions of the morphometric characters on carapace length (C) in all statistical analyses. Therefore possible statistical differences found among populations do not simply reflect body size differences (ADAMS and BEACHY, 2001). All regressions were strongly correlated (males: $106 < n < 129$, $r > 0.47$; females: $110 < n < 127$, $r > 0.50$) and highly significant ($p < 0.001$).

One-way ANOVA was used to select the measurements, which differ significantly among populations; only these variables were included in multivariate analyses. We assessed both morphometric variations among populations and the *species* and *origin* effects, performing multivariate analysis of variance (MANOVA) followed up using discriminant analysis (FIELD, 2000). The classification success rate was evaluated basing on the percentage of individuals correctly classified in the original sample. Finally, in order to compare distances among groups we calculated Euclidean distances between each pair of centroids on all significant discriminant axes. Statistical differences were detected with a level of significance $p < 0.05$; all statistical calculations were performed in SPSS software.

RESULTS

Genetic data

The mtDNA 16S sequence alignment (456 nucleotides) showed 22 different haplotypes of *A. pallipes* and *A. italicus* (9 from the province of Alessandria) and one specimen of *A. torrentium* as outgroup (Figure 2). The nucleotide divergence among the haplotypes examined, expressed as percentage, is reported in the Table III.

In Alessandria province we can confirm the presence of *A. pallipes* (haplotypes: LG2, LG15, LG34, LG19, LEM1B) and *A. italicus* (haplotypes: SB, LG20, GAM2B, LAV2B). Average of genetic variation (calculated as p distance = number of substitution / total number of nucleotides examined expressed in percentage) between the two species sampling is $3.5 \pm 0.32\%$.

The SB haplotype, which could be ascribed to the taxon *Austropotamobius italicus carinthiacus*, seems widespread in 80% of the watercourses of the province. In Visone River we found a population with two different haplotypes (LAV2B, GAM2B), which form part of the subclade 3 relative to the ssp. *Austropotamobius italicus meridionalis*.

The haplotypes LG2, LG15, LG34, LG19, LEM1B which form part of clade B (*Austropotamobius pallipes*) characterise only four populations from watercourses sited in the southern part of the province close to the Ligurian Apennine. In two of the last ones (Lagoscuro and Mulino Brooks) we found syntopy between *A. pallipes* and *A. italicus*.

Syntopic populations

The genetic identification of all 47 crayfishes collected in the three stretches of the Lagoscuro Brook shows the dominance (64.3%) of *italicus* in the terminal stretch (A) and

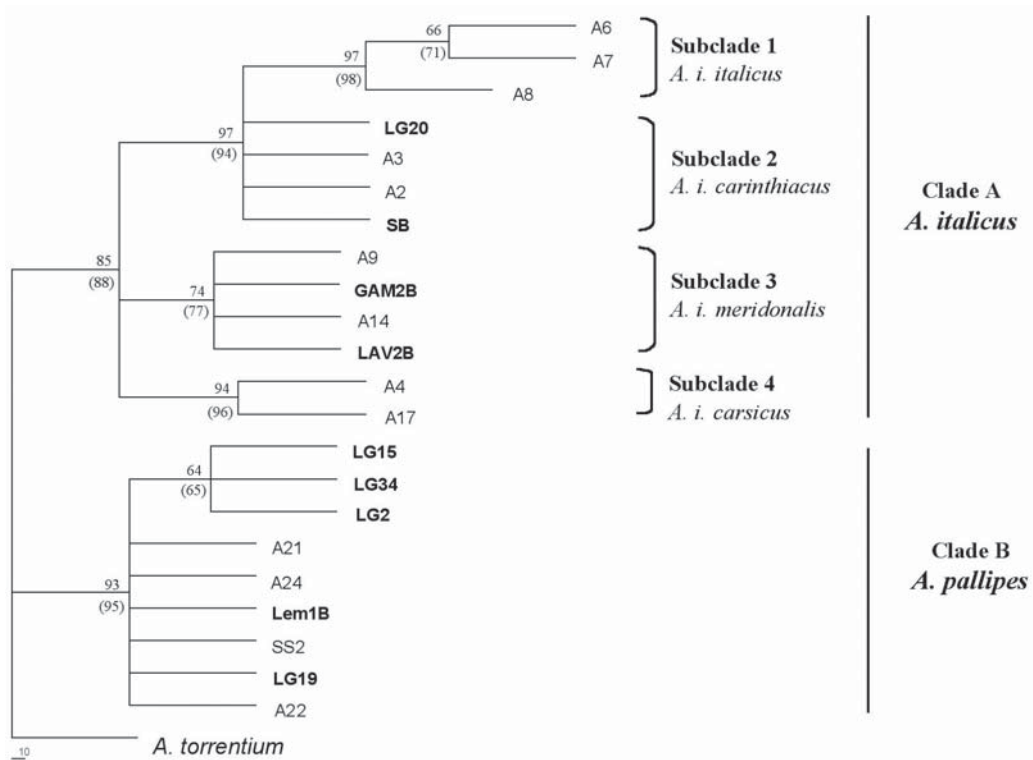


Figure 2

Molecular phylogeny of white-clawed crayfish inferred by MP analysis (consensus tree) and NJ analysis of a 456 bp mitochondrial DNA fragment (16S mtDNA). *A. torrentium* was used to root the tree. Bootstrap values are given above nodes (1,000 replications; only confidence values higher than 50% are shown in the tree). Alessandria haplotypes (AL) are written in bold character: *pallipes* AL vs. *italicus carinthiacus* AL = 3.9%, *italicus carinthiacus* AL vs. *italicus meridionalis* AL = 1.8%, *italicus meridionalis* AL vs. *pallipes* AL = 3.3%.

Figure 2

Phylogénie moléculaire de l'écrevisse à pattes blanches déduite par le biais des analyses MP (arbre consensus) et NJ des fragments de ADN mitochondrial d'une longueur de 456 pb (16S ADNmt). *A. torrentium* a été utilisé pour enracciner l'arbre. Les valeurs de bootstrap figurant au-dessus des nœuds (1 000 répliquions ; seuls les indices de confiance supérieurs à 50 % sont illustrés sur l'arbre). Les haplotypes d'Alessandria (AL) sont écrits en caractère gras : *pallipes* AL vs. *italicus carinthiacus* AL = 3,9 %, *italicus carinthiacus* AL vs. *italicus meridionalis* AL = 1,8 %, *italicus meridionalis* AL vs. *pallipes* AL = 3,3 %.

the sudden inversion of abundance ratio in favour of *pallipes* in the other two stretches (80% in B and 83.3% in C) (Figure 3).

The average of nucleotidic divergence between *A. pallipes* and *A. italicus* was found to be 3.9%, thus showing a clear separation between the two species. The two haplotypes, belonging to *A. italicus carinthiacus* (SB, LG20) and found within the population, present a minimum difference of one deletion. We could highlight four different haplotypes of *A. pallipes*; LG15, LG34, LG2 are present with similar frequencies and minimum differences of one or more deletion; LG19 (6.25% of the whole "*pallipes* sample") is spaced out from the preceding ones for 0.22%.

Table III

Pairwise mtDNA 16S divergence (calculated as ρ -distance = number of substitutions / total number of nucleotides examined and expressed as percentage) between *Austropotamobius* haplotypes. *Alessandria* haplotypes (in bold): LG2, LG15, LG19, LG20, LG34, SB, LEM1B, LAV2B, GAM2B. Italian haplotypes downloaded from GenBank: A2, A3, A4, A6, A7, A8, A9, A14, A17, A21, A22, A24 (FRATINI et al., 2005) and SS2 (ZACCARA et al., 2004). Insertions/deletions gaps were treated as missing data. Torr = *Austropotamobius torrentium*.

Tableau III

Divergence « deux à deux » du ADNmt 16S (calculé comme ρ -distance = nombre de substitutions / nombre total de nucléotides examinés et exprimé en pourcentage) entre les haplotypes de *Austropotamobius*. Haplotypes d'*Alessandria* (en gras): LG2, LG15, LG19, LG20, LG34, SB, LEM1B, LAV2B, GAM2B. Les haplotypes italiens téléchargés de GenBank : A2, A3, A4, A6, A7, A8, A9, A14, A17, A21, A22, A24 (FRATINI et al., 2005) et SS2 (ZACCARA et al., 2004). Les lacunes « insertions/délétions » sont traitées comme des données manquantes. Torr = *Austropotamobius torrentium*.

Haplotype	LG15	LG2	LG34	LG19	SS2	LEM1B	A22	A24	A9	LAV2B	A14	LG20	SB	GAM2B	A6	A7	A8	A2	A3	A4	A17	A21	Torr	
LG15	/																							
LG2	0.00	/																						
LG34	0.00	0.00	/																					
LG19	0.22	0.22	0.22	/																				
SS2	0.66	0.66	0.66	0.44	/																			
LEM1B	0.22	0.22	0.22	0.00	0.44	/																		
A22	0.22	0.22	0.22	0.00	0.44	0.00	/																	
A24	0.44	0.44	0.44	0.22	0.66	0.22	0.22	/																
A9	3.54	3.53	3.54	3.31	3.31	3.32	3.31	3.53	/															
LAV2B	3.54	3.53	3.54	3.31	3.31	3.32	3.31	3.53	0.40	/														
A14	3.32	3.31	3.31	3.09	3.01	3.10	3.09	3.31	0.22	0.22	/													
LG20	3.98	3.97	3.98	3.75	3.01	3.76	3.75	3.97	1.99	1.99	1.77	/												
SB	3.98	3.98	3.98	3.76	3.31	3.76	3.76	3.98	1.99	1.99	1.77	0.00	/											
GAM2B	3.32	3.32	3.32	3.10	3.09	3.10	3.10	3.32	0.22	0.22	0.00	1.77	1.77	/										
A6	4.42	4.41	4.42	4.19	3.97	4.20	4.19	3.97	3.09	3.31	2.87	1.10	1.10	2.87	/									
A7	4.64	4.63	4.64	4.41	4.19	4.42	4.41	4.19	3.31	3.31	3.09	1.32	1.32	3.09	0.22	/								
A8	4.20	4.19	4.20	3.97	3.75	3.98	3.97	3.75	2.87	2.87	2.65	0.88	0.88	2.65	0.22	0.44	/							
A2	3.98	3.97	3.97	3.74	3.31	3.76	3.74	3.97	1.99	1.99	1.77	0.00	0.00	1.77	1.10	1.32	0.88	/						
A3	3.98	3.97	3.98	3.75	3.31	3.76	3.75	3.97	1.99	1.99	1.77	0.00	0.00	1.77	1.10	1.32	0.88	0.00	/					
A4	3.76	3.76	3.76	3.54	3.09	3.54	3.54	3.76	1.77	1.77	1.54	2.43	2.43	1.55	3.09	3.31	2.87	2.43	2.43	/				
A17	3.31	3.30	3.31	3.08	2.65	3.09	3.08	3.31	1.76	1.76	1.54	2.42	2.43	1.54	3.08	3.30	2.87	2.42	2.42	0.44	/			
A21	0.44	0.45	0.45	0.22	0.67	0.22	0.22	0.44	3.55	3.56	3.33	3.99	4.00	3.34	4.43	4.65	4.21	3.99	3.99	3.78	3.33	/		
Torr	8.45	8.43	8.45	8.21	8.65	8.22	8.21	8.43	8.21	8.43	8.43	9.10	9.11	8.44	9.99	10.20	9.77	9.10	9.10	9.10	9.09	8.47	/	

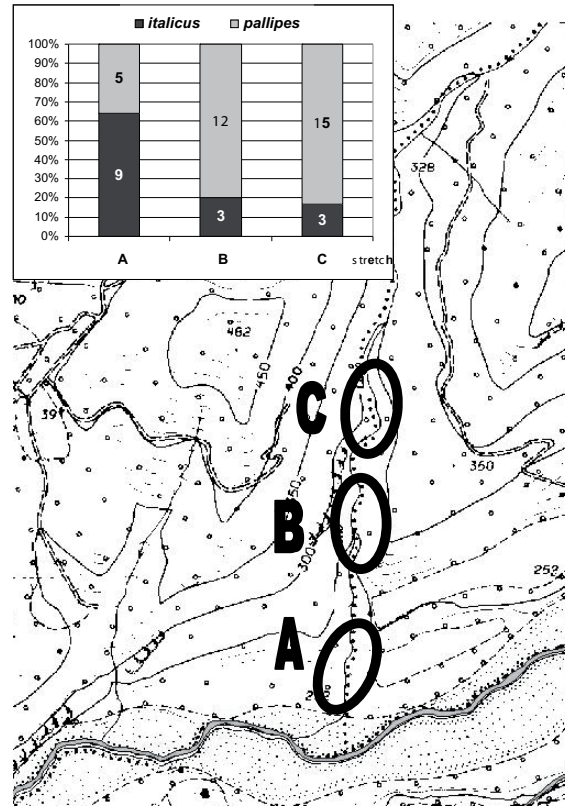


Figure 3
Location of the three stretches in the Lagoscuro Brook and distribution of the two species *A. pallipes* and *A. italicus*.

Figure 3
Localisation des trois secteurs du ruisseau Lagoscuro et distribution des deux espèces, *A. pallipes* et *A. italicus*.

Morphometric data

We analyzed a sample of 256 individuals. Species, sexes, origin and mean total lengths (A) as reported in Table IV.

The one-way ANOVA results show the absence of significant differences between the populations (by origin and by species) in males for the parameters S, X and Z (= cephalotorax window width, branchio-cardiac groove length and telson length) and in females for X and B (branchio-cardiac groove length and cephalotorax length); therefore they are excluded from subsequent analyses. All the other variables have highly significant differences (Table V).

The multivariate analysis of variance (MANOVA) in both sexes shows morphometric features significantly different according to their origins, but it does not give any possibility of discriminating *pallipes* from *italicus* crayfishes collected in the Lagoscuro Brook, for some morphometric features (Table VI).

The difference as regards the origin, the only significant effect from MANOVA, is also confirmed by the discriminant analysis, which gives very high reclassification percentages for both sexes; the individuals, on average, were correctly reclassified in 94.3% and 94.5% of cases in males and in females, respectively. Also for the Lagoscuro Brook the percentages of correctly reclassified cases reach 83.3% in males and 93.8% in females (Table VII).

Table IV

Total length of the samples, divided into species, geographic origin and sexes.

Table IV

Longueur totale des échantillons, répartis par espèce, par origine géographique et par sexe.

		Total length (mm)					
		males			females		
species	origin	mean	range	N	mean	range	N
<i>A. italicus</i>	Angiassi	81.639	67.83-103.65	19	80.451	68.47-92.54	15
	Lagoscuro	58.298	45.32-88.89	5	53.668	46.37-60.10	5
	Reponde	79.775	69.29-93.58	14	76.695	66.20-95.24	21
	Schizzola	79.748	65.08-107.88	37	83.648	64.98-108.67	20
Total		78.802	45.32-107.88	75	78.011	46.37-108.67	61
<i>A. pallipes</i>	Lagoscuro	59.319	45.28-72.39	9	63.772	48.65-74.91	12
	Lemme	69.911	47.75-97.13	45	66.534	42.39-92.55	54
	Total	68.145	45.28-97.13	54	66.032	42.39-92.55	66

Table V

One-way ANOVA results in both sexes. Values in bold character do not show significant differences.

Table V

Résultats pour les deux sexes de l'ANOVA à un facteur. Les valeurs en caractère gras ne montrent pas de différences significatives.

Morphological parameters	Males			Females		
	df among populations; df within populations	F	Sig.	df among populations; df within populations	F	Sig.
B	5; 123	6.083	0.000	5; 119	1.105	0.361
D	5; 123	7.209	0.000	5; 121	2.920	0.016
E	5; 101	3.235	0.009	5; 104	22.394	0.000
G	5; 100	4.271	0.001	5; 104	21.012	0.000
I	5; 101	7.195	0.000	5; 104	23.696	0.000
L	5; 121	6.087	0.000	5; 120	6.997	0.000
M	5; 123	13.847	0.000	5; 120	9.210	0.000
N	5; 123	5.109	0.000	5; 121	3.852	0.003
O	5; 123	10.777	0.000	5; 121	3.200	0.009
P	5; 123	16.991	0.000	5; 121	22.867	0.000
Q	5; 123	4.270	0.001	5; 121	6.686	0.000
R	5; 123	15.175	0.000	5; 121	9.642	0.000
S	5; 123	0.885	0.493	5; 121	5.343	0.000
T	5; 123	4.341	0.001	5; 121	7.060	0.000
U	5; 123	6.904	0.000	5; 121	10.020	0.000
V	5; 123	9.605	0.000	5; 121	12.637	0.000
X	5; 123	1.347	0.249	5; 121	2.052	0.076
Y	5; 123	22.001	0.000	5; 121	14.271	0.000
Z	5; 123	1.284	0.275	5; 121	12.047	0.000

Table VI

Results from MANOVA comparisons of the six crayfish populations, in both sexes.

Tableau VI

Résultats de la comparaison MANOVA de six populations d'écrevisses, pour les deux sexes.

	Effect	Wilks' λ	F	Hypothesis df	Error df	P
males	Intercept	0.766	1.604	16	84	0.085
	species	0.836	1.030	16	84	0.435
	origin	0.037	6.831	64	331.121	0.000
females	Intercept	0.671	2.507	17	87	0.003
	species	0.826	1.078	17	87	0.388
	origin	0.060	5.286	68	343.731	0.000

Table VII

Crayfish reclassification results into origin groups based on discriminant analysis, in both sexes. Rows are the original sample group and columns the reclassification group; percentage column represents reclassification into each population relative to the population of origin.

Tableau VII

Re-classification des résultats des écrevisses en groupes d'origine basée sur l'analyse discriminante, pour les deux sexes. Les lignes correspondent à l'échantillon de base et les colonnes au groupe re-classifié ; le pourcentage des colonnes représente la classification de chaque population par rapport à la population d'origine.

	Origin	Angiassi	Lagoscuro	Reponte	Schizzola	Lemme	n	%
Males	Angiassi	17	0	0	1	0	18	94.4
	Lagoscuro	2	10	0	0	0	12	83.3
	Reponte	0	1	12	0	0	13	92.3
	Schizzola	0	1	1	19	0	21	90.5
	Lemme	0	0	0	0	41	41	100.0
Females	Angiassi	13	2	0	0	0	15	86.7
	Lagoscuro	0	15	1	0	0	16	93.8
	Reponte	0	0	19	0	0	19	100.0
	Schizzola	1	0	0	7	1	9	77.8
	Lemme	0	1	0	0	49	50	98.0

Males: 94.3% of original grouped cases correctly classified.

Females: 94.5% of original grouped cases correctly classified.

All the four discriminant functions give a significant contribution to group separation. For males, the first two discriminant functions explain conjointly the 71.9% of the total variance; the morphometric characters mainly loadings on these functions are I, P and Y (claw width, rostrum width and abdominal length). Instead, for females, the

first discriminant function explains by itself the 69.5% of the total variance; E, G, I (claw variables) and P (rostrum width) have largest absolute correlation with the function. An overview of the results is provided in Table VIII.

The dispersal graphs (Figure 4) show how the groups are well separated and how it is possible to identify some morphometric elements as typical of the populations according to their origin. In particular, the males from the Angiassi, Lagoscuro, Schizzola and Reponte Brooks tend to develop wider claws and longer abdomen than those from Lemme Torrent, which, on the contrary, can be distinguished for the narrower rostrum basis. In the females the same differences are obvious as regards all the characters of the claws.

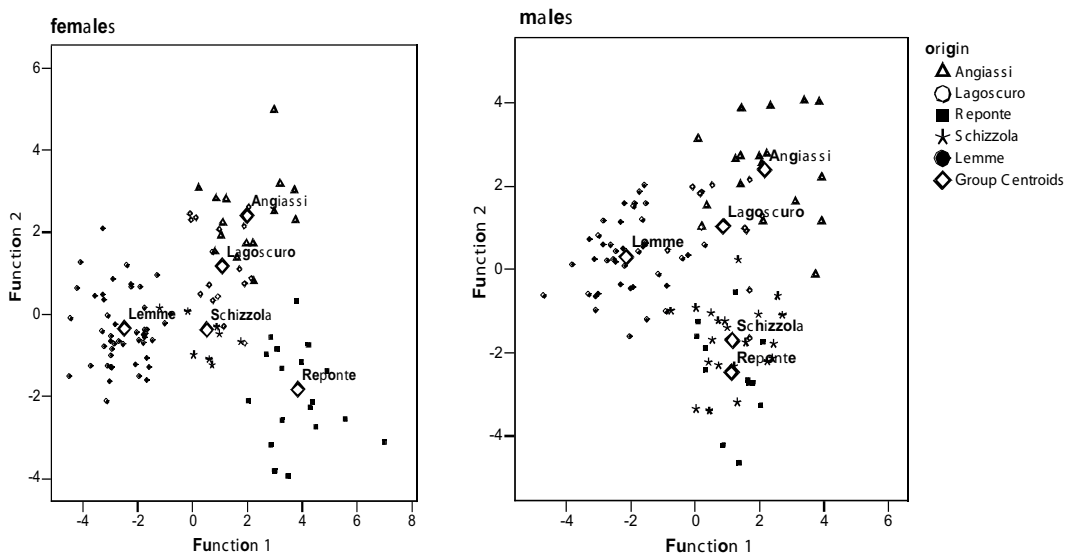


Figure 4
Plots of the scores of individuals according to the first two discriminant functions, generated from 16 (in males) and 17 (in females) morphometric variables.

Figure 4
Représentation des valeurs des individus selon les deux premières fonctions discriminantes, générées par les variables morphométriques, 16 pour les mâles et 17 pour les femelles.

In order to compare distances among groups, a Euclidean distances matrix was created (Table IX) between each pair of centroids calculated from the coordinates of the centroids on discriminant axes, which resulted, to be significant, for both sexes.

These morphometric distances show how both the males and the females from Lagoscuro Brook are closer to those from Angiassi and farther from the Reponte males and females.

DISCUSSION

The Alessandria province is confirmed as a contact zone with the presence of the two species *A. pallipes* and *A. italicus*, as already proposed by NASCETTI *et al.* (1997), using allozymes and, more recently, using mtDNA 16S gene (ZACCARA *et al.*, 2004; FRATINI *et al.*, 2005). The mean nucleotidic divergence ($3.5 \pm 0.32\%$) shows a sharp

Table VIII

The structure matrix shows the correlations of each morphometric character with each discriminant function, in both sexes. The eigenvalue and the percentage of variance explained are given below.

Tableau VIII

La structure de la matrice montre les corrélations de chaque caractère morphométrique avec chaque fonction discriminante, pour les deux sexes. La valeur propre et le pourcentage de variance expliquée sont donnés dessous.

Males	Discriminant functions			
	1	2	3	4
I	0.312	0.029	- 0.076	0.030
G	0.250	- 0.051	0.020	0.096
E	0.171	- 0.122	0.038	- 0.107
Y	0.323	0.409	- 0.136	0.028
D	0.093	0.313	- 0.050	0.181
U	0.023	0.264	- 0.085	- 0.097
V	- 0.107	0.263	- 0.077	0.184
N	0.035	0.258	- 0.019	0.136
T	0.052	0.164	- 0.132	- 0.050
R	0.241	- 0.176	0.509	0.296
O	- 0.019	0.200	0.421	0.315
B	0.004	0.126	- 0.160	0.103
M	- 0.233	- 0.125	- 0.243	0.644
P	- 0.351	0.207	0.305	0.505
L	0.200	- 0.003	0.273	0.334
Q	- 0.009	0.046	- 0.238	0.242
Eigenvalue	3.258	2.596	1.741	0.552
% variance	40.0	31.9	21.4	6.8
Females	1	2	3	4
E	0.402	0.116	- 0.018	0.071
G	0.389	0.138	0.038	- 0.028
I	0.384	0.256	0.120	0.333
Z	- 0.204	0.150	- 0.144	0.105
Y	0.070	0.539	- 0.031	0.156
U	- 0.182	0.282	- 0.044	0.062
T	- 0.159	0.261	- 0.010	- 0.034
O	- 0.009	0.251	0.139	- 0.159
D	0.086	0.230	- 0.021	0.042
N	0.028	0.164	- 0.127	- 0.002
R	- 0.105	- 0.065	0.563	0.076
L	0.092	0.127	0.525	0.165
P	- 0.364	0.118	0.391	0.199
Q	0.176	- 0.095	- 0.229	0.040
M	- 0.165	- 0.138	0.174	0.547
V	- 0.217	0.153	- 0.027	0.297
S	- 0.144	- 0.051	0.015	- 0.278
Eigenvalue	6.455	1.724	0.679	0.426
% variance	69.5	18.6	7.3	4.6

Table IX

Euclidean distances between each pair of centroids calculated on all discriminant axes; the distances are given above and below the diagonal for females and males, respectively.

Table IX

Distances euclidiennes entre chaque paire de centroïdes calculées sur la base de tous les axes discriminants ; les distances sont données en dessous de la diagonale pour les femelles et en dessus pour les mâles.

Origin	Angiassi	Lagoscuro	Reponte	Schizzola	Lemme
Angiassi	–	2.84	4.70	4.19	5.33
Lagoscuro	3.41	–	4.37	3.72	4.18
Reponte	5.59	4.43	–	4.70	6.51
Schizzola	4.69	4.45	4.53	–	4.11
Lemme	4.82	3.86	4.97	4.32	–

separation between the two species in agreement with previous *Austropotamobius* phylogeny study (GRANDJEAN *et al.*, 2000) where the two species appear separated from a range of variability comprised between 3.5-6.5%. In almost all the watercourses *A. italicus carinthiacus* (haplotype SB) is present, while in the south portion we can find some brooks with *A. pallipes* (Lemme and S. Bartolomeo Brooks). Moreover, in the Visone Torrent we could assess the presence of *A. italicus* ssp. *meridionalis* (haplotypes LAV2B, GAM2B), whose distribution area covers only Central Southern Italy. Subsequently, historical investigations have brought us to ascribe the presence of these allochthonous haplotypes in the Alessandria territory to an introduction by man, which occurred in the 1930s' (MINISTERO DELL'AGRICOLTURA E DELLE FORESTE, 1931). The high frequency of a single haplotype dominating in the whole province (SB, *A. italicus carinthiacus*), compared with the greater genetic heterogeneity of *A. pallipes*, could be explained by a rapid expansion of *A. italicus* from the Italian glacial refugia with the effect of a diffusion of genetic homogeneity (HEWITT, 1996) or by the result of a more recent bottleneck phenomenon (ZACCARA *et al.*, 2004).

Based on allozymatic analyses, NASCETTI *et al.* (1997) found an overlapping zone between *A. pallipes* and *A. italicus* in the Ligurian Apennine, where the morphological studies of FROGLIA (1978) had already stressed the existence of populations with intermediate features between the two species. The results of the genetic analyses, based here on a larger number of samples, confirm the presence of both species in, at least, two watercourses already pointed out by ZACCARA *et al.* (2004). The preliminary collecting of samples from these brooks, conducted randomly, shows that the two species dwell in the same stretch of the watercourse, living in syntopy. The results of the subsequent investigations, aimed at defining the distributions of *pallipes* and *italicus* in the Lagoscuro Brook, show a kind of gradient in the abundance ratios of the two species only in the terminal tract (about 200 m) of the watercourse, while they stress a clear inversion of the presence percentages in the upstream stretches.

The scarcity of studies on similar situations makes particularly difficult the interpretation of this picture. A study conducted in France on the overlapping zone between *A. pallipes* and *A. astacus* allowed to identify a brook where the two species coexist naturally, but they do not live in syntopy (LACHAT and LAURENT, 1988); the different features of the habitat settle *A. astacus* downstream and *A. pallipes* upstream, with some ten metres of no man's land between them. Also in the Lagoscuro Brook

A. pallipes seems to prefer the higher part of the watercourse (80% in stretch B and 83.3% in C), where the torrent features are more evident; the distribution of the two species is however continuous along the watercourse, without interruptions. A sympatry situation concerning two congeneric species of crayfish is present in the Außerfern (Austria), where *A. torrentium* is autochthonous in Archbach (below Mühl near Reutte), while *A. pallipes* was introduced in the first half of the XX century in Lake Plansee and subsequently developed a large population which further spread to Heiterwanger See and the out-flowing Archbach (above the Stuiben waterfalls) (FÜREDER and MACHINO, 1995; 1999). In some Bavarian river systems *A. astacus* and *A. torrentium* may be found occupying the same waterbody, with very short zone of overlap (BOHL, 1988; HOLDICH, 2002).

The simultaneous presence in the territory of Alessandria province (and in NW Po River hydrographic catchments basin) of both species and the syntopy phenomenon are of difficult interpretation without a real geographic barrier that limits a natural dispersion. Some hypotheses have been proposed in preceding studies (NASCETTI *et al.*, 1997; ZACCARA *et al.*, 2004; FRATINI *et al.*, 2005). This phenomenon may be due to events of human translocation (SPITZY, 1978) already at the end of the Middle Ages, when crayfish represented an important food source (ARRIGNON, 1996); or it could be the result of ancient patterns of penetration of *A. pallipes*, which could have crossed the watershed of the Ligurian Apennine in different points, after local events of plate tectonics which formed the catchment of the watercourse sources in the Apennines (CATTAUTO, CENCETTI and GREGORI, 1988). The Alessandria province would therefore represent a natural contact zone between the two species, following the expansion of their distribution areas from the respective glacial refugia; the syntopic populations could be considered then as relict populations.

No hybridisation phenomenon (NASCETTI *et al.*, 1997) between the two species has been found in syntopic populations of the same river system, but we have no information so far concerning the populations which live in the same watercourse. Even if the mean nucleotidic divergence between *A. pallipes* and *A. italicus* from Lagoscuro Brook (3.9%) expresses a clear separation between the two species, possible hybridisations cannot be assessed with the matrilineal molecular marker used in this study (mtDNA). Even morphometrical analyses do not provide reliable information on hybridisation, due to high morphological variability, as this study shows. Our preliminary attempt of interspecific reproduction experiment in the laboratory (unpublished data) suggests a probable reproductive isolation; these results, however, require further research with more numerous samples and the finding of possible natural hybrids by biparental molecular marker, i.e. the microsatellites (FETZNER and CRANDALL, 2002).

Even if the genetic results show a sharp separation between the two species *pallipes* and *italicus*, in the Lagoscuro Brook where they coexist, from the morphometric results it was impossible to point out a character useful for the accurate specific recognition, especially in the field research. Also LÖRTSCHER *et al.* (1997), in a study on populations of *A. pallipes* complex sampled in the five larger basins of the Alpine region, have found results conflicting between morphological and genetic analyses; the observation of different individuals with intermediate or atypical morphological characters contrasts with the finding of three well differentiated groups at genetic level.

Moreover, it is evident (MANOVA and discriminant analysis) that crayfishes from various watercourses are morphometrically different and well distinct, as already found in French populations of *A. pallipes* (GRANDJEAN *et al.*, 1997a). The morphometric characters involved in such phenotypic distinction concern, mainly, claws and rostrum basis, in both sexes. When intraspecific comparisons on *A. astacus*, *A. leptodactylus* e *P. leniusculus* are concerned, the shape of claw seems to be more discriminating character compared to shape of rostrum (AGERBERG, 1988).

Furthermore the matrix of morphometrical distances confirms that there is no greater difference for *pallipes* from Lemme Torrent than for *italicus* from the other sites. The restricted sampling area which we investigated in the present study, does not allow us to find biogeographical explanations, but the results obtained seem however to indicate a dominating role of the environmental features peculiar for each watercourse, in comparison with the genetic position of the populations taken into consideration.

REFERENCES

- ADAMS D.C., BEACHY C.K., 2001. Historical explanations of phenotypic variation in the plethodontid salamander *Gyrinophilus porphyriticus*. *Herpetologica*, 57(3), 353-364.
- AGERBERG A., 1988. Multivariate analysis of morphometric variation in three species of freshwater crayfish. *Freshwater Crayfish*, 7, 19-28.
- ALBRECHT H., 1982. Das System der europäischen Flusskrebse (Decapoda, Astacidae): Vorschlag und Begründung. *Mitt. Hamb. Zool. Mus. Inst.*, 79, 187-210.
- ARRIGNON J., 1996. *L'écrevisse et son élevage*. Technique & Documentation, Paris, III^e ed.
- ATTARD J., VIANET R., 1985. Variabilité génétique et morphologique de cinq populations de l'écrevisse européenne *Austropotamobius pallipes* (Lereboullet, 1858) (Crustacea, Decapoda). *Can. J. Zool.*, 63, 2933-2939.
- BOHL E., 1988. Comparative studies on crayfish brooks in Bavaria (*Astacus astacus* L., *Austropotamobius torrentium* Schr.). *Freshwater Crayfish*, 7, 287-294.
- BOTT R., 1950. Die Flusskrebse Europas. *Abhandlungen der Senckenbergischen Naturforschenden Gesellschaft*, 483, 1-36.
- BOTT R., 1972. Besiedlungsgeschichte und Systematik der Astaciden West-Europas unter besonderer Berücksichtigung der Schweiz. *Revue suisse de zoologie*, 79, 387-408.
- BRODSKY S.Y., 1983. On the systematic of palearctic crayfishes (Crustacea, Astacidae). *Freshwater Crayfish*, 5, 464-470.
- CATTAUTO C., CENCETTI C., GREGORI L., 1988. Lo studio dei corsi d'acqua minori dell'Italia Appenninica come mezzo di indagine sulla tettonica del Plio/Pleistocene. *Bull. Mus. St. Nat. Lunigiana*, 6-7, 7-10.
- FELSENSTEIN J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39, 783-791.
- FETZNER J.W. Jr., CRANDALL K.A., 2002. Genetic Variation. In: D.M. HOLDICH (ed.): *Biology of freshwater crayfish*. Blackwell Science Oxford, pp. 291-326.
- FIELD A., 2000. *Discovering statistics using SPSS for Windows*. SAGE Publications Ltd, London.
- FRATINI S., ZACCARA S., BARBARESÌ S., GRANDJEAN F., SOUTY-GROSSET C., CROSA G., GHERARDI F., 2005. Phylogeography of the Threatened crayfish (genus *Austropotamobius*) in Italy: implications for its taxonomy and conservation. *Heredity*, 94, 108-118.
- FROGLIA C., 1978. *Decapodi (Crustacea Decapoda)*. Guide per il riconoscimento delle specie animali delle acque interne italiane. 4. C.N.R. AQ/1/9, Verona.
- FÜREDER L., MACHINO Y., 1995. Record of the white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) from Plansee (Tyrol, Austria). *Berichte des Naturwissenschaftlich-Medizinischen Vereins in Innsbruck*, 82, 211-219.

- FÜREDER L., MACHINO Y., 1999. Past and present crayfish situations in Tyrol (Austria and Northern Italy). *Freshwater Crayfish*, 12, 751-764.
- GRANDJEAN F., ROMAIN D., SOUTY-GROSSET C., MOCQUARD J.P., 1997a. Size at sexual maturity and morphometric variability in three populations of *Austropotamobius pallipes pallipes* (Lereboullet, 1858) according to a restocking strategy. *Crustaceana*, 70 (4), 454-468.
- GRANDJEAN F., ROMAIN D., AVILA-ZARZA C., BRAMARD M., SOUTY-GROSSET C., MOCQUARD J.P., 1997b. Morphometry, sexual dimorphism and size at maturity of the white-clawed crayfish *Austropotamobius pallipes pallipes* (Lereboullet) from a wild French population at Deux-Sèvres (Decapoda, Astacidae). *Crustaceana*, 70 (1), 31-44.
- GRANDJEAN F., HARRIS D.J., SOUTY-GROSSET C., CRANDALL K.A., 2000. Systematic of the European endangered crayfish species *Austropotamobius pallipes* (Decapoda: Astacidae). *J. Crust. Biol.*, 20, 522-529.
- GRANDJEAN F., BOUCHON D., SOUTY-GROSSET C., 2002. Systematic of the European endangered crayfish species *Austropotamobius pallipes* (Decapoda: Astacidae) with a re-examination of the status of *Austropotamobius berndhauseri*. *J. Crust. Biol.*, 22, 677-681.
- HEWITT G.M., 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of Linnean Society*, 58, 247-276.
- HOLDICH D.M., 2002. Background and Functional Morphology. In: D.M. HOLDICH (ed.): *Biology of freshwater crayfish*. Blackwell Science Oxford, pp. 3-29.
- KARAMAN M.S., 1962. Ein Beitrag zur Systematik der Astacidae (Decapoda). *Crustaceana*, 3, 173-191.
- LACHAT G., LAURENT P.J., 1988. The habitats of *Astacus astacus* L. and *Austropotamobius pallipes* Lere. in the Morvan. *Freshwater Crayfish*, 7, 61-68.
- LAURENT P.J., SUSCILLON M., 1962. Le écrevisses en France. *Annales de la Station Centrale d'Hydrobiologie*, 9, 335-395.
- LÖRTSCHER M., STUCKI T.P., CLALÜNA M., SCHOLL A., 1997. Phylogeographic structure of *Austropotamobius pallipes* populations in Switzerland. *Bull. Fr. Pêche Piscic.*, 347, 649-661.
- MINISTERO DELL'AGRICOLTURA E DELLE FORESTE, 1931. *La pesca nei mari e nelle acque interne d'Italia. Notiziario tecnico e legislativo e repertorio dell'industria e del commercio dei prodotti pescherecci*. Vol 2. Istituto Poligrafico dello Stato, Roma.
- MORI M., MANDICH A., ISOLA G., 1991. Morfometria e maturità sessuale di due popolazioni di *Austropotamobius pallipes fulcisianus* (Ninni) (Crustacea, Astacidae) della provincia di Genova. *Boll. Mus. Ist. Biol. Univ. Genova*, 54-55, 49-58.
- 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. *S. It. E. Atti*, 18, 205-208.
- NEGRI A., CAPRÌ F., DONDERO F., VIARENGO A., MALACARNE G., NARDI P.A., BO T., 2003. Use of genetic markers in the endangered freshwater crayfish *Austropotamobius* spp. In: 22nd Conference of European Society for Comparative Physiology and Biochemistry (Alessandria, 14-18 Dicembre 2003).
- PALUMBI S., MARTIN A., ROMANO S., McMILLAN W.O., STICE L., GRABOWSKI G., 1991. *The Simple Fools Guide to PCR, version II*. University of Hawaii, Honolulu.

- SANTUCCI F., IACONELLI M., ANDREANI P., CIANCHI R., NASCETTI G., BULLINI L., 1997. Allozyme diversity of European freshwater crayfish of the genus *Austropotamobius*. *Bull. Fr. Pêche Piscic.*, 347, 663-676.
- SINT D., DALLA VIA J., FÜREDER L., 2004. Morphological variations in *Astacus astacus* L. and *Austropotamobius pallipes* (Lereboullet). In: Craynet, 3rd Thematic Meeting "European native crayfish in relation to land-use and habitat deterioration with a special focus on *Austropotamobius torrentium*" (Innsbruck, September 8-11, 2004); Poster.
- SPITZY R., 1978. The prehistoric man as a possible crayfish transplanter. *Freshwater Crayfish*, 4, 221-225.
- STREISSL F., HÖDL W., 2002. Growth, morphometrics, size at maturity, sexual dimorphism and condition index of *Austropotamobius torrentium* Schrank. *Hydrobiologia*, 477, 201-208.
- SWOFFORD D.L., 1998. *PAUP* – Phylogenetic Analysis Using Parsimony (* and Other Methods)*. Version 4. Sinauer Associates; Sunderland, MA.
- THOMPSON J.D., GIBSON T.J., PLEWNIAK F., JEANMOUGIN F., HIGGINS D.G., 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 24, 4876-4882.
- 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. *Biological Conservation*, 120, 1-10.