ALLOZYME DIVERSITY OF EUROPEAN FRESHWATER CRAYFISH OF THE GENUS *AUSTROPOTAMOBIUS*.

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ABSTRACT

Data are reported on genetic variation at 30 enzyme loci in *Austropotamobius* crayfish from France, England, Italy, Spain, and the Balkans. Three population groups were detected, corresponding to *A. torrentium*, *A. pallipes* and *A. italicus* (*sensu* KARAMAN, 1962). Reproductive isolation between these three taxa was demonstrated in the field. Populations from England, France, and north-western Italy belong to *A. pallipes sensu stricto*, those from the rest of Italy, Spain, western Slovenia and north-western Croatia belong to *A. italicus*, whereas *A. torrentium* was found in the Balkans up to the Italian border. An average D_{Nei} of 0.30 was found between *A. pallipes* and *A. italicus*, while the average genetic distance between these two species and *A. torrentium* was $D_{Nei} = 0.77$. Populations of *A. italicus* from Spain and north-central Apennines were found to be genetically closely related ($D_{Nei} = 0.05$), not supporting a subspecific rank for these populations (*A. i. lusitanicus*). Marked interpopulation genetic diversity was observed both within *A. italicus* (average $F_{ST} = 0.80$, D_{Nei} up to 0.18), and within *A. torrentium* (average $F_{ST} = 0.73$, D_{Nei} up to 0.14), comparable to that previously reported for *A. pallipes*. Such genetic heterogeneity appears mainly related to range fragmentations and subsequent recolonizations from multiple *refugia* during the last glacial events. Overall low values of genetic variability were found in the samples tested (*e.g.* H_e from 0 to 0.05), with the highest values in larger sized populations from less disturbed areas (*e.g.* in *A. italicus* from Spain and Slovenia). The genetic erosion observed in *Austropotamobius* populations stresses the need to restore their genetic variability, *e.g.* by controlled restocking, for successful programs of recovery and management of these endangered crayfish.

Key-words : allozymes, freshwater crayfish, *Austropotamobius*, molecular systematics, genetic heterogeneity, genetic erosion, restocking, conservation strategies.

DIVERSITÉ ALLOZYMIQUE DES ÉCREVISSES EUROPÉENNES DU GENRE *AUSTROPOTAMOBIUS*.

RÉSUMÉ

Cette étude présente les résultats sur la variation génétique de 30 loci enzymatiques chez les écrevisses d'eau douce du genre *Austropotamobius* provenant de France, d'Angleterre, d'Italie, d'Espagne et des Balkans. Trois groupes de population ont été identifiés, correspondant à *A. torrentium*, *A. pallipes* et *A. italicus* (*sensu* KARAMAN, 1962). L'isolement reproductif dans la nature entre ces trois espèces a été démontré. Les populations d'Angleterre, de France et d'Italie nord-occidentale appartiennent à *A. pallipes sensu stricto*, celles du reste de l'Italie, de l'Espagne, de la Slovénie occidentale et de la Croatie nord-occidentale appartiennent à *A. italicus*, tandis qu'*A. torrentium* a été trouvé dans les Balkans jusqu'au bord de l'Italie. La distance génétique moyenne entre *A. pallipes* et *A. italicus* est D_{Nei} = 0,30, tandis qu'entre ces deux espèces et *A. torrentium* elle est D_{Nei} = 0,77. Les populations d'*A. italicus* d'Espagne et des Apennins centre-septentrionaux sont génétiquement proches (D_{Nei} = 0,05), rejetant ainsi l'idée d'une sous-espèce séparée pour ces populations (*A. i. lusitanicus*). Une remarquable diversité génétique a été observée parmi différentes populations géographiques d'*A. italicus* (F_{ST} moyen = 0,80, D_{Nei} jusqu'à 0,18) et d'*A. torrentium* (F_{ST} moyen = 0,73, D_{Nei} jusqu'à 0,14), comparable à celle reportée précédemment pour *A. pallipes*. Cette hétérogénéité génétique semble être principalement due aux dernières glaciations, induisant une fragmentation géographique et des recolonisations successives à partir de refuges multiples. Une faible variabilité génétique a été trouvée dans l'ensemble des populations étudiées (H_e de 0 à 0,05). Les populations ayant les valeurs les plus élevées sont aussi les plus nombreuses et correspondent à celles qui vivent dans des zones moins affectées par l'activité humaine (par exemple *A. italicus* d'Espagne et de Slovénie). L'érosion génétique observée chez les populations d'*Austropotamobius* montre qu'il est nécessaire de restaurer leur variabilité génétique (par exemple par des réintroductions contrôlées), pour une sauvegarde et une gestion réussie de ces écrevisses menacées d'extinction.

Mots-clés : allozymes, écrevisses d'eau douce, *Austropotamobius*, systématique moléculaire, hétérogénéité génétique, érosion génétique, réintroduction, stratégies de sauvegarde.

INTRODUCTION

Three subspecies are recognized by some authors in the European white-clawed crayfish *Austropotamobius pallipes : A. p. pallipes* from France, part of Switzerland and Austria, and the British Isles ; *A. p. lusitanicus* from Spain and Portugal and *A. p. italicus* from Italy, part of Switzerland, and the Dalmatian coast (BOTT, 1950, 1972 ; HOLTHUIS, 1978). The last two taxa are considered as belonging to a distinct species, *A. italicus*, by other authors (KARAMAN, 1962, 1963 ; BRODSKI, 1983). A morphologically well differentiated species, assigned by some authors to a distinct subgenus, is the stone crayfish *A. torrentium*, inhabiting the Balkans and part of central Europe.

Genetic variation at 30 enzyme loci was analyzed in populations of *A. pallipes sensu lato* from France, England, Italy, Spain, Slovenia and Croatia and of *A. torrentium* from northern Balkans. Aim of the study was to investigate the following points : **I)** the taxonomic rank of *A. italicus* ; **II)** the genetic divergence among *Austropotamobius* populations and taxa ; **III)** the levels of intrapopulation genetic variability in *Austropotamobius* populations. The last point also tests the hypothesis that genetic erosion is involved in the dramatic decline of European freshwater crayfish. The systematic outcomes of the results obtained and their relevance to management strategies of these endangered crayfish are discussed.

MATERIAL AND METHODS

Field populations of European freshwater crayfish of the genus *Austropotamobius* were genetically investigated, including 31 samples belonging to *A. pallipes sensu lato*, (2 from France, 1 from England, 23 from Italy, 2 from Spain, 2 from Slovenia, 1 from Croatia) and 4 of *A. torrentium* from north-eastern Italy, Slovenia and Croatia. Sample locations are given in Table I. Standard horizontal starch gel electrophoresis was carried out on muscle tissue from single thoracic appendixes, frozen in liquid nitrogen, of living specimens which were released immediately after amputation. Twenty-one enzymes, encoded by 30 putative loci, were analyzed. The electrophoretic techniques used are summarized in Table II. Although the number of loci could have easily been enhanced by testing other tissues (*e.g.*, hepatopancreas, gonads, brain, *etc.*), we preferred to use a non-destructive method for these endangered crayfish (thoracic appendixes can regenerate), still allowing the analysis of a number of loci sufficiently high to estimate genetic diversity (*cf.* AVISE, 1994).

Table I

Sample codes (c), geographic origin, and number of specimens analyzed (n) in *Austropotamobius* populations studied : e = altitude (m a.s.l.) ; UK = United Kingdom ; F = France ; E = Spain ; I = Italy ; S = Slovenia ; C = Croatia.

Tableau I

Echantillons (c), origine géographique, et nombre d'individus analysés (n) chez les populations d'*Austropotamobius* : e = altitude (m) ; UK = Royaume-Uni ; F = France ; E = Espagne ; I = Italie ; S = Slovénie ; C = Croatie.

c	Geographic origin	e	River system	Region	n
A. pallipes sensu lato					
1	Norfolk	20	Yare	East Anglia (UK)	9
2	Causse du Larzac	400	Garonne	Aveyron (F)	33
3	Montagne de l'Espérou	600	Hérault	Gard (F)	21
4	Puertos de Beseitle	800	Ebro	Teruel (E)	52
5	Vega de Granada	1000	Guadalquivir	Granada (E)	23
6	Alpi Marittime	730	Po	Liguria (I)	24
7	Alpi Cozie	300	Po	Piemonte (I)	34
8	Alpi Cozie	500	Po	Piemonte (I)	30
9	Serra di Ivrea	320	Po	Piemonte (I)	32
10	Prealpi Pennine	500	Po	Piemonte (I)	14
11	Alto Monferrato	190	Po	Piemonte (I)	14
12	Prealpi Bergamasche	450	Po	Lombardia (I)	62
13	Prealpi Carniche	700	Tagliamento	Friuli (I)	5
14	Prealpi Carniche	250	Tagliamento	Friuli (I)	29
15	Rivignano	20	Stella	Friuli (I)	25
16	Appennino Ligure-Emiliano	550	Po	Piemonte	38
17	Appennino Ligure-Emiliano	450	Po	Emilia (I)	38
18	Appennino Tosco-Emiliano	300	Po	Emilia (I)	17
19	Pratomagno	500	Arno	Toscana (I)	37
20	Selva del Lamone	300	Fiora	Lazio (I)	22
21	Monti Sabatini	300	Mignone	Lazio (I)	32
22	Monti Reatini	900	Tevere	Lazio (I)	30
23	Monti Sabini	450	Tevere	Lazio (I)	32
24	Monti della Laga	600	Vomano	Abruzzi (I)	11
25	Monti Simbruini	730	Tevere	Abruzzi (I)	12
26	Vallo di Diano	450	Sele	Campania (I)	8
27	Monti della Maddalena	650	Agri	Basilicata (I)	8
28	Massiccio del Pollino	650	Coscile	Calabria (I)	48
29	Kolovrat	120	Soca	Banjska Planota (S)	38
30	Brkini	700	Reka	Kras (S)	47
31	Cicaria	300	Mirna	Istra (C)	22
A. torrentium					
32	Alpi Carniche	1000	Danube	Friuli (I)	11
33	Juliske Alpe	320	Danube	Dolenjsko (S)	20
34	Luksici	250	Danube	Medvednica (C)	11
35	Lokve	600	Mrzlica	Gorski Kotar (C)	20

Table II

The enzymes scored, listed with their international code number (EEC), encoding loci, electrophoretic migration conditions and staining references (Ref.). + = anodal ; - = cathodal.

Tableau II

Enzymes étudiés, avec leur code numérique international (EEC), loci correspondants, conditions de migration éléctrophorétique et référence bibliographique (Ref.) de la technique de coloration. + = anodique, - = cathodique.

Enzymes	EEC	Encoding Loci	Migration	Buffer system	V/cm	Run (hours)	Ref.
α-Glycerophosphate dehydrogenase	1.1.1.8	*α-Gpdh*	+	1,6	8	4	d
Lactate dehydrogenase	1.1.1.28	*Ldh*	+	1	8	4	a
Malate dehydrogenase	1.1.1.37	*Mdh-1*	+	4	8	4	b
		Mdh-2	+				
Isocitrate dehydrogenase	1.1.1.42	*Idh-1*	+	3,7	8	4	b
		Idh-2	+				
6-Phosphogluconate dehydrogenase	1.1.1.44	*6Pgdh*	+	4	8	4	b
Octanol dehydrogenase	1.1.1.73	*Odh*	+	5	8	5	c
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	*Gapdh*	+	3	8	4	d
Xanthine dehydrogenase	1.2.1.37	*Xdh*	+	3	8	4	c
NADH dehydrogenase	1.6.99.3	*NADHdh*	+	1	8	4	a
Superoxide dismutase	1.15.1.1	*Sod-1*	+	1,2,3	8	4	c
		Sod-2					
Aspartate aminotransferase	2.6.1.1	*Aat-1*	+	5	8	6	b
		Aat-2	+,–				
Alanine aminotransferase	2.6.1.2	*Alat-1*	+	5	8	5	f
		Alat-2	+				
Phosphoglycerate kinase	2.7.2.3	*Pgk*	+	3	8	4	f
Esterase	3.1.1.1	*Est-1*	+	2	8	3	d
		Est-2	+				
Peptidase (Leu-Gly-Gly)	3.4.11	*Pep-B1*	+	6	8	5	f
		Pep-B2	+				
		Pep-B3	+				
Peptidase (Leu-Ala)	3.4.11	*Pep-C*	+	3	8	5	f
Aldolase	4.1.2.13	*Ald*	+	3	8	4	d
Carbonic anhydrase	4.2.1.1	*Ca*	+	3	8	4	e
Mannose-6-phosphate isomerase	5.3.1.8	*Mpi*	+	2	8	3	e
Glucose-6-phosphate isomerase	5.3.1.9	*Gpi*	+	2	8	3	c
Phosphoglucomutase	5.4.2.2	*Pgm-1*	+	1	8	4	a
		Pgm-2	+				

*Buffer systems are the following : 1) discontinuous tris/citrate (POULIK, 1957) ; 2) continuous tris/citrate (SELANDER *et al.*, 1971) ; 3) tris/versene/borate (BREWER and SING, 1970) ; 4) tris/versene/maleate (BREWER and SING, 1970) ; 5) discontinuous lithium/borate (SOLTIS *et al.*, 1983). **Staining references : a) BREWER and SING (1970) ; b) SHAW and PRASAD (1970) ; c) SELANDER *et al.* (1971) ; d) AYALA *et al.* (1972) ; e) HARRIS and HOPKINSON (1976) ; f) RICHARDSON *et al.* (1986).

For each population sample, the following parameters of genetic variability were estimated, using BIOSYS-I software (SWOFFORD and SELANDER, 1981) : **1)** percent of polymorphic loci, using the 0.99 (P_{99}) and 0.95 (P_{95}) criteria ; **2)** mean number of alleles per locus (A) ; observed (H_o) and expected (H_e) mean heterozygosity per locus. Partitioning of intra- and interpopulation genetic diversity was calculated from variable loci using the F statistics by WRIGHT (1943, 1951). Genetic divergence was estimated with the indices by NEI (1972, D_{Nei}) and ROGERS (1972, modified by WRIGHT, 1978, D_T).

Genetic relationships among populations and taxa were represented using : **1)** cluster analysis, by neighbour-joining (SAITOU and NEI, 1987) and UPGMA (SNEATH and SOKAL, 1973) methods from D_{Nei} values, using PHYLIP software (FELSENSTEIN, 1995) ; **2)** multidimensional scaling ordination (MDS, GUTTMAN, 1968) from D_T values, using SYSTAT software (WILKINSON and LELAND, 1989).

RESULTS

Five out of the 30 loci analyzed (*Mdh-2, Idh-2, Sod-1, Alat-1, Mpi*) were found monomorphic for the same allele in all the *Austropotamobius* samples tested. *A. pallipes sensu lato* (samples 1-31) and *A. torrentium* (samples 32-35) showed distinct alleles at 11 loci *(Mdh-1, Odh, NADHdh, Aat-1, Aat-2, Alat-2, Pgk, Est-1, PepB-2, PepC, Ca*, see Table III) and highly differentiated frequencies at three loci (*α-Gpdh, Pgm-2, PepB-3*), with complete lack of gene exchange and an average D_{Nei} value of 0.77 (range 0.60 - 0.91).

Table III

Loci found diagnostic between *Austropotamobius pallipes s. s.*, *A. italicus* and *A. torrentium*.

Tableau III

Loci diagnostiqués entre *Austropotamobius pallipes s. s.*, *A. italicus* et *A. torrentium*.

	A. italicus	***A. torrentium***
A. pallipes s. s.	*α-Gpdh, Odh, Aat-2, Alat-2, PepB-1, PepC, Pgm-1*	*α-Gpdh, Mdh-1, Odh, NADHdh, Aat-1, Aat-2, Alat-2, Pgk, Est-1, PepB-1, PepB-2, PepB-3, PepC, Ca, Pgm-1, Pgm-2*
A. italicus	——	*Mdh-1, Odh, NADHdh, Aat-1, Aat-2, Alat-2, Pgk, Est-1, PepB-2, PepC, Ca*

Within *A. pallipes s. l.*, 5 additional loci (*Aat-1, Est-1, PepB-2, Ald, Ca*), were found to be monomorphic for the same allele ; another 9 (*Ldh, Idh-1, 6Pgdh, G3pdh, Xdh, NADHdh, Est-2, Gpi, Pgm-2*) were polymorphic, sharing the same most common allele ; at another 7 loci (*α-Gpdh, Odh, Aat-2, Alat-2, PepB-1, PepC, Pgm-1,* Table III) distinct fixed alleles were found in different populations, allowing distinction of two genetically well differentiated groups. The first one includes the samples from England (1), France (2, 3), and north-western Italy (6-10), the second one includes the remaining samples from Italy (11-28), Slovenia (29, 30), Croatia (31) and Spain (4, 5). These two groups correspond to *A. pallipes* and *A. italicus sensu* KARAMAN (1962). Their geographic location is shown on the map in Figure 1.

No F_1, F_N hybrids, or backcrosses were detected in the samples tested of *A. pallipes s. s.* and *A. italicus*, even when collected in the same river system. Individuals showing introgression at one or a few loci were found in populations from Liguria and Piedmont (not included in the present study), indicating past hybridization events (paleointrogression) but

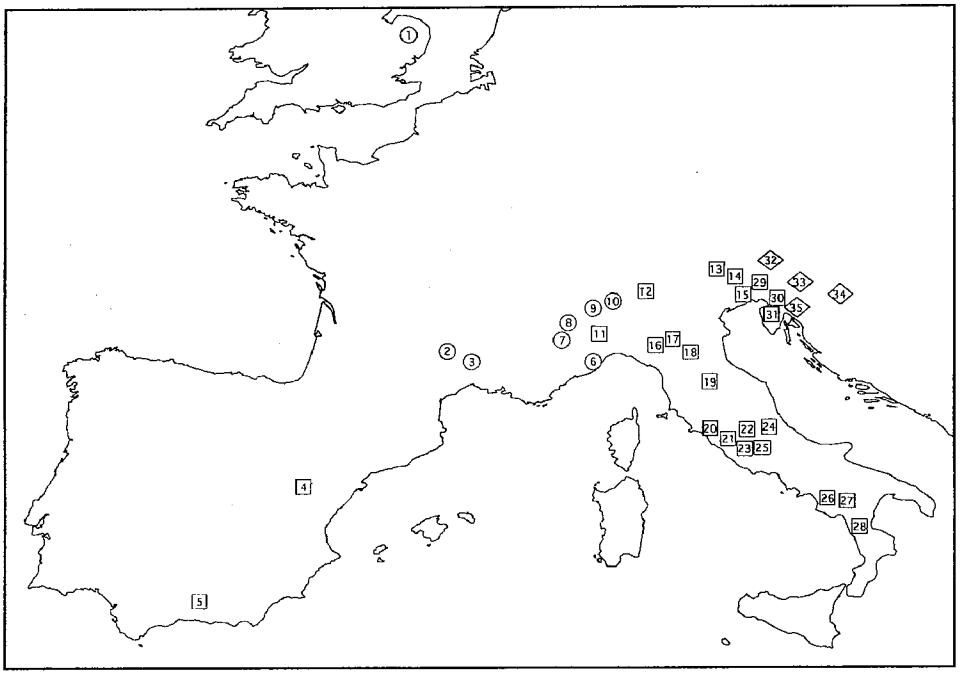

Figure 1

Geographic location of the population samples studied of European freshwater crayfish of the genus *Austropotamobius*, assigned according to genetic data to *A. pallipes sensu stricto* (circles), *A. italicus* (squares) and *A. torrentium* (diamonds). For sample codes, see Table I.

Figure 1

Localisation géographique des échantillons d'écrevisses européennes du genre *Austropotamobius*, classés sur la base des données génétiques comme : *A. pallipes sensu stricto* (cercles), *A. italicus* (carrés) et *A. torrentium* (losanges). Pour le code des échantillons, voir Tableau I.

lack of present gene flow (in preparation ; see Discussion). The average genetic distance between *A. pallipes s. s.* and *A. italicus* (paleointrogressed populations were not included in calculations) is $D_{Nei} = 0.30$ (range 0.26 - 0.40). The genetic relationships between populations of these two species and *A. torrentium* are shown by the UPGMA cluster in Figure 2 ; a similar topology was obtained by neighbour joining (not shown). A spatial picture of the genetic relationships among *A. pallipes s. s.* and *A. italicus* populations is given by the plot of the first two dimensions of a MDS analysis (Figure 3). These two methods provide a consistent patterning.

Marked genetic heterogeneity was found within *A. italicus*, with differentiated frequencies at several loci (*Mdh-1, Sod-2, Aat-2, Pgk, PepB-1, PepB-3, PepC*). The values of F_{ST} per locus range from 0.02 to 0.96 (average 0.80) ; their distribution is plotted in Figure 4. Accordingly, a broad range of genetic distances was detected among populations (D_{Nei} from 0 to 0.18), partially related to their geographic distances. Populations 4 and 5 from the Iberian Peninsula proved to be poorly differentiated genetically from those from the north-central Apennines (11, 16-19), joining in the same cluster (Figure 2). Another cluster includes the samples from Latium (20-23), Abruzzi (24, 25), and southern Italy (26-28) ; a third cluster joins samples from Lombardy (12), Friuli (13, 14, 15) and north-western Croatia (31) ; sample 30 from western Slovenia clusters with the last two groups. Finally, a sample from the Italian-Slovenian border (29) appears to be genetically well differentiated from the others (average $D_{Nei} = 0.13$). Genetic differentiation was found also at a lower geographic scale, apparently due to local loss of polymorphism by genetic drift (*e.g.* in the samples 16, 17 and 31). An overall picture of genetic relationships among populations of *A. italicus* is summarized in Figures 2 and 3.

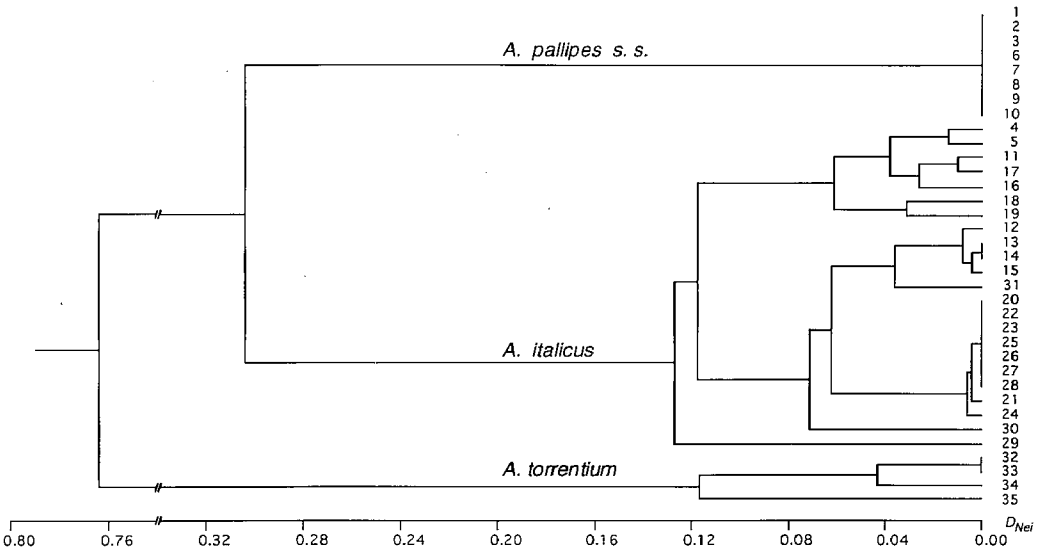

Figure 2

UPGMA dendrogram based on NEI's (1972) standard genetic distance values (D_{Nei}), showing the genetic relationships among European freshwater crayfish populations of *Austropotamobius pallipes sensu stricto*, *A. italicus* and *A. torrentium*. For sample codes, see Table I.

Figure 2

Dendrogramme UPGMA obtenu à partir des distances génétiques de NEI (1972, D_{Nei}), montrant les relations génétiques des populations d'écrevisses européennes des espèces *Austropotamobius pallipes sensu stricto*, *A. italicus* et *A. torrentium*. Pour le code des échantillons, voir Tableau I.

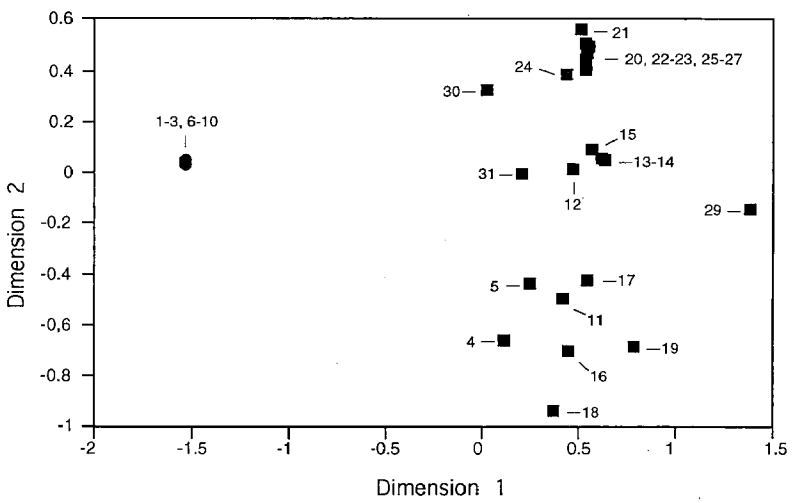

Figure 3

Plot of the first two dimensions of a MDS analysis (GUTTMAN, 1968) based on ROGERS' (1972, modified by WRIGHT, 1978) genetic distance values (D_T), showing the genetic relationships among European freshwater crayfish populations of *Austropotamobius pallipes sensu stricto* (circles) and *A. italicus* (squares). For sample codes, see Table I.

Figure 3

Représentation des deux premières dimensions d'une analyse MDS (GUTTMAN, 1968) obtenue à partir des valeurs des distances génétiques de ROGERS (1972, modifié par WRIGHT, 1978, D_T), montrant les relations génétiques des populations d'écrevisses européennes des espèces *Austropotamobius pallipes sensu stricto* (cercles) et *A. italicus* (carrés). Pour le code des échantillons, voir Tableau I.

The population samples of *A. pallipes s. s.* from England (1), southern France (2, 3) and north-western Italy (6-10), showed no genetic differentiation (average D_{Nei} = 0.00, *cf.* Figure 2), with a very low F_{ST} value (0.09, Figure 4). This finding appears to be related to the geographic origin of these populations, since *A. pallipes* samples from other locations (in Ireland, northern and southern France) showed a marked genetic heterogeneity, with values of D_{Nei} and F_{ST} comparable to those observed within *A. italicus* (Figure 4 ; ATTARD and VIANET, 1985).

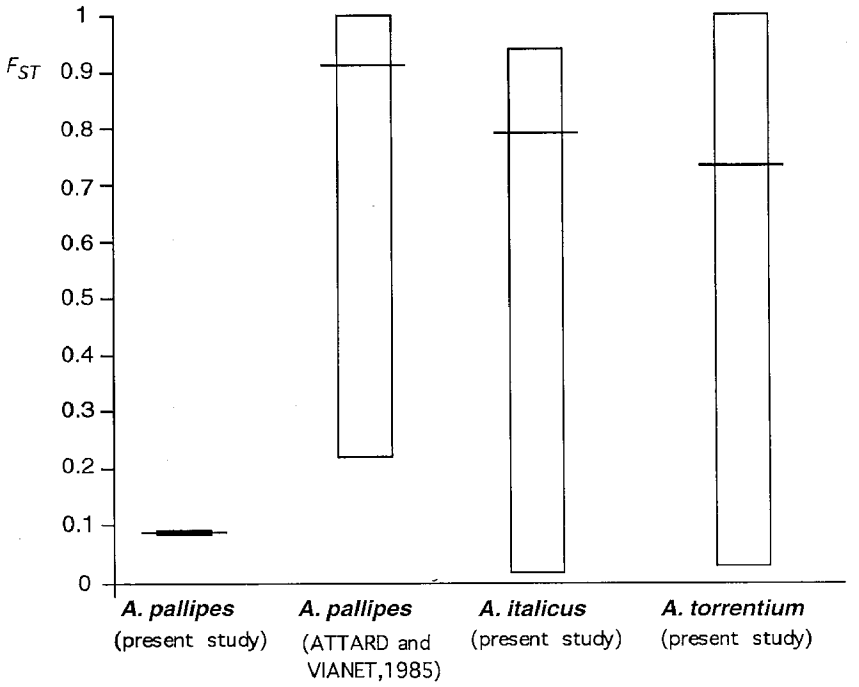

Figure 4

Ranges (boxes) and average values (lines) of F_{ST} per locus observed for populations of *Austropotamobius pallipes sensu stricto*, *A. italicus* and *A. torrentium*. Data from present study and ATTARD and VIANET (1985).

Figure 4

Intervalles (rectangles) et moyennes (barres) des valeurs de F_{ST} par locus, observés pour les populations d'*Austropotamobius pallipes sensu stricto*, d'*A. italicus* et d'*A. torrentium*. Données provenant de cette étude et de celle d'ATTARD et VIANET (1985).

Genetic heterogeneity was also detected within *A. torrentium*, with differentiated frequencies at the loci *Aat-1*, *Est-2*, *Ald*, and *Ca*. The values of F_{ST} per locus range from 0.02 to 1.00 (average 0.73), as shown in Figure 4. The values of D_{Nei} range from 0.001 to 0.14 ; the samples most closely related are those from the Friuli-Slovenia border (32) and Slovenia (33), whereas the most differentiated one is that from western Croatia (35).

Genetic variability parameters estimated for *Austropotamobius* populations, both from our study and from ATTARD and VIANET (1985) data, are given in Table IV. A virtual lack of polymorphism (H_e < 0.01, P_{95} = 0) at the loci tested was observed in a substantial number of samples, *i.e.* those of *A. torrentium* from Slovenia (33), of *A. pallipes* from north-western Italy (6-10), northern France (Haute-Saône), southern France (3), Corsica, England (1), and of *A. italicus* from north-central Apennines (16, 20), and southern Italy (26, 27). Low values of genetic variability were found in various other samples, with H_e ranging from 0.01 to 0.02 and P_{95} from 0.03 to 0.07. The highest values (H_e = 0.03-0.05) were found in samples of *A. italicus* from Spain (4, 5), Slovenia (30), Italian Prealps (12, 15), central Apennines (24), and of *A. torrentium* from Croatia (34).

Table IV

Parameters of genetic variability in European crayfish of the genus *Austropotamobius*. *A* = mean number of alleles per locus ; P_{95}, P_{99} = percent of polymorphic loci, at the 0.95 and 0.99 criteria, respectively ; H_o = observed and H_e = expected mean heterozygosity per locus. S.E. = standard error. Source of data (Ref.) : a = present study, b = ATTARD and VIANET (1985).

Tableau IV

Estimation de la variabilité génétique chez les écrevisses du genre *Austropotamobius*. *A* = nombre moyen d'allèles par locus ; P_{95}, P_{99} = pourcentage de loci polymorphes, avec les critères du 0.95 et 0.99, respectivement ; H_o = hétérozygotie observée et H_e = hétérozygotie théorique moyennes par locus. S.E. = erreur standard. Origine des données (Réf.) : a = cette étude, b = celle d'ATTARD et VIANET (1985).

Sample code	*A*±S.E.	P_{95}	P_{99}	H_o±S.E	H_e±S.E.	Ref.
A. pallipes s.s.						
1 (UK)	1.0	0.0	0.0	0.000	0.000	a
2 (F)	1.0	3.3	3.3	0.007±0.007	0.007±0.007	a
3 (F)	1.0	0.0	0.0	0.000	0.000	a
6 (I)	1.0	0.0	0.0	0.000	0.000	a
7 (I)	1.0	0.0	0.0	0.000	0.000	a
8 (I)	1.0	0.0	0.0	0.000	0.000	a
9 (I)	1.0	0.0	0.0	0.000	0.000	a
10 (I)	1.0	0.0	0.0	0.000	0.000	a
average	**1.0**	**0.4**	**0.4**	**0.001**	**0.001**	
Haute-Saône (F)	*1.0*	*0.0*	*0.0*		*0.000*	*b*
Gard (F)	*1.1*	*5.6*	*5.6*		*0.013*	*b*
Hérault (F)	*1.1*	*5.6*	*5.6*		*0.020*	*b*
Haute-Corse (F)	*1.0*	*0.0*	*0.0*		*0.000*	*b*
Ireland	*1.1*	*5.6*	*5.6*		*0.026*	*b*
A. italicus						
4 (E)	1.1±0.1	10.0	10.0	0.036±0.021	0.035±0.021	a
5 (E)	1.2±0.1	10.0	13.3	0.051±0.029	0.053±0.029	a
11 (I)	1.1±0.1	3.3	3.3	0.020±0.020	0.025±0.025	a
12 (I)	1.2±0.1	16.7	23.3	0.046±0.022	0.048±0.022	a
13 (I)	1.1±0.1	6.7	10.0	0.010±0.006	0.010±0.006	a
14 (I)	1.1±0.1	6.7	10.0	0.009±0.006	0.009±0.006	a
15 (I)	1.2±0.1	10.0	13.3	0.032±0.019	0.033±0.020	a
16 (I)	1.0	0.0	3.3	0.003±0.003	0.003±0.003	a
17 (I)	1.1±0.1	3.3	10.0	0.012±0.009	0.012±0.009	a
18 (I)	1.1	3.3	6.7	0.006±0.004	0.006±0.004	a
19 (I)	1.1	3.3	6.7	0.004±0.003	0.006±0.005	a
20 (I)	1.0	0.0	3.3	0.003±0.003	0.003±0.003	a
21 (I)	1.1±0.1	6.7	10.0	0.021±0.014	0.023±0.016	a
22 (I)	1.2±0.1	3.3	13.3	0.016±0.012	0.018±0.014	a
23 (I)	1.2±0.1	6.7	20.0	0.018±0.008	0.017±0.008	a
24 (I)	1.1±0.1	10.0	13.3	0.039±0.021	0.037±0.020	a
25 (I)	1.1	6.7	6.7	0.019±0.015	0.019±0.015	a
26 (I)	1.0	0.0	0.0	0.000	0.000	a
27 (I)	1.0	0.0	0.0	0.000	0.000	a
28 (I)	1.1±0.1	3.3	10.0	0.010±0.006	0.010±0.006	a
29 (S)	1.0	3.3	3.3	0.015±0.015	0.015±0.015	a
30 (S)	1.2±0.1	13.3	16.7	0.038±0.020	0.042±0.022	a
31 (C)	1.0	3.3	3.3	0.018±0.018	0.017±0.017	a
average	**1.1**	**5.6**	**9.1**	**0.018**	**0.019**	
A. torrentium						
32 (I)	1.1±0.1	6.7	10.0	0.013±0.008	0.018±0.011	a
33 (S)	1.1	0.0	6.7	0.005±0.004	0.005±0.004	a
34 (C)	1.1±0.1	10.0	13.3	0.041±0.023	0.039±0.022	a
35 (C)	1.1±0.1	3.3	10.0	0.015±0.011	0.017±0.013	a
average	**1.1**	**5.0**	**10.0**	**0.018**	**0.020**	

DISCUSSION

Allozyme analysis has proved to be a powerful approach to evidence genetic structuring of *Austropotamobius* crayfish. Three genetically well differentiated groups were detected among the populations tested : one from England, France and north-western Italy, corresponding to *A. pallipes sensu stricto* ; a second one from the rest of Italy, Spain, north-western Balkans, corresponding to *A. italicus* (including *lusitanicus*) ; a third, most differentiated one, from north-eastern Italian border and the Balkans, corresponding to *A. torrentium.*

As above-mentioned, *A. italicus* has been considered either as a subspecies of *A. pallipes* (BOTT, 1950, 1972 ; HOLTHUIS, 1978) or as a distinct species (including *lusitanicus*), by KARAMAN (1962, 1963) and BRODSKI (1983). ALBRECHT (1982), on the basis of a clinal variation at some morphological characters in samples from Tessin and northern Italy, considered *pallipes* and *italicus* only as variations of *A. pallipes.* Populations with some morphological intermediacy between *A. italicus* and *A. pallipes* were detected in Liguria and Piedmont (Italy) by FROGLIA (1978). The genetic study of such populations (in preparation) showed lack of F_1, F_n hybrids or backcrosses, but a low level of introgression. The absence of present gene exchange between *A. pallipes* and *A. italicus* populations, even when located a few kilometers apart in the same river system, indicates that the introgression observed is the result of past hybridization events that took place when the two taxa came into secondary contact, after the last ice-age. Accordingly, the reproductive isolation between *A. pallipes* and *A. italicus* has apparently been completed only after such secondary contact, presumably due to selection against hybrids (reinforcement). The specific status of *A. pallipes* and *A. italicus*, proposed by KARAMAN (1962), appears confirmed by these data.

Samples from Spain (4, 5) were found to be genetically closely related (D_{Nei} = 0.05) to those from north-central Apennines (11, 16-19). This rules out that our Spanish populations belong to a distinct subspecies (*A. i. lusitanicus*). This finding is supported by the closer relatedness detected at mtDNA level between samples of *A. pallipes s. l.* from Spain and Slovenia with respect to those from France and England (GRANDJEAN *et al.*, 1997a ; SOUTY-GROSSET *et al.*, 1997, this same volume).

The genetic relatedness found between populations of *A. italicus* from Spain and north-central Apennines suggests that gene flow took place between them up to recently ; accordingly, the present range of *A. italicus* would be the relict of a broader one, including southern France. The disjunction of such range was presumably caused by the spread of *A. pallipes* to the south, as suggested by KARAMAN (1962). The higher competitive ability of *A. pallipes* is shown by its recent spread in north-western Italy, where it has displaced *A. italicus.* The disjunct range of *A. italicus* in Italy and Spain is paralleled by the geographic distribution of the chamois *Rupicapra pyrenaica*, which now lives only in the Cantabrics, the Pyrenees and Abruzzi Apennines. Also in this case, range disjunction was due to the spread of a more effective competitor, *R. rupicapra* from eastern Europe, which excluded *R. pyrenaica* from most of its previous range, as documented by fossil records (NASCETTI *et al.*, 1985 ; LOVARI, 1989).

A marked genetic heterogeneity was observed within *A. italicus*, with the following main population groups, from : **I)** Spain and north-central Apennines ; **II)** Latium, Abruzzi and southern Italy ; **III)** north-eastern Italy and north-western Croatia ; **IV)** Italian-Slovenian border, with a single population. The last one may be either a relict population, or part of a taxon living in areas genetically not studied so far ; it might correspond to *A. i. carsicus* described by KARAMAN (1962) from Dubrovnik surroundings.

In the case of *A. pallipes*, our samples proved to be genetically similar ; on the other hand, genetic heterogeneity was detected among samples from Ireland, northern and southern France by ATTARD and VIANET (1985). Further genetic heterogeneity was found in Switzerland, with a complex picture, possibly involving the taxon *A. berndhauseri* BOTT, 1950 (LÖRTSCHER *et al.*, 1997, this same volume). Further studies appear needed, with a common set of genetic markers, and involving populations from parts of *A. pallipes* geographic distribution not investigated so far.

As to *A. torrentium*, two population clusters were detected in the north-western Balkans ; however, a large part of the range of this species remains to be studied genetically, both from central Europe and central-southern Balkans.

The interpopulation genetic diversity found in *Austropotamobius* crayfish appears to be mainly related to range fragmentations and subsequent recolonizations from multiple *refugia* during the last glacial events, as well as, at a lower geographic scale, to genetic drift. These phenomena are now well documented in various organisms, both plants and animals (HEWITT, 1996).

Passive transport by man of crayfish is considered a frequent event (ALBRECHT, 1983 ; HOLDICH, 1988 ; GRANDJEAN *et al.*, 1997a). It may explain the lack of genetic divergence between our samples of *A. pallipes* from England and southern France, as well as between those from southern France and Corsica observed by ATTARD and VIANET (1985). Nevertheless, native populations of crayfish existed in England, as shown by the pleistocene fossil records found in Essex and Lincolnshire (BELL, 1920 ; KARAMAN, 1962). Crayfish transport obviously does not rule out that native populations were already present in a given region. For example, crayfish were transported to Ireland in the 19th century (ALBRECHT, 1983), but the existence of native crayfish in this island is suggested by the finding of a population genetically well differentiated (ATTARD and VIANET, 1985). As to the Iberian Peninsula, native and introduced populations seem to coexist (BALSS, 1925 ; HOLDSWORTH, 1880 ; HUXLEY, 1879 ; MATEUS, 1937) ; the samples from Spain analyzed in the present study are genetically related to those from Italian Apennines ; however, they show a higher genetic variability and a number of alleles not detected elsewhere (private alleles), which would rule out their introduction by man.

A very low genetic variability was found in many *Austropotamobius* populations, with different markers : allozymes by the present study, by ATTARD and VIANET (1985) and LÖRTSCHER *et al.* (1997, this same volume) ; and mtDNA by GRANDJEAN *et al.* (1997a, b) and SOUTY-GROSSET *et al.* (1997, this same volume). Such low intrapopulation genetic variation appears to be due to different events, often interacting, such as range fragmentations, massive extinctions, population crashes, reduction of suitable habitats, pollution, overfishing, spread of parasitic and infective diseases, introduction of outcompeting alloctonous species, *etc.* (MANCINI, 1986, 1988 ; HOLDICH, 1988 ; SOUTY-GROSSET *et al.*, 1997, this same volume ; GRANDJEAN *et al.*, 1997a, b).

Crayfish populations inhabiting areas less affected by man are generally more numerous and genetically more variable, such as *A. italicus* from some locations in Spain, Slovenia, Italy, and *A. torrentium* from part of Croatia. Accordingly, genetic erosion is apparently involved in the massive decline of *Austropotamobius*. Successful programs of recovery and management of these endangered crayfish should involve *ad hoc* measures to restore their genetic variability, *e.g.* by controlled restocking from genetically variable populations. To this purpose, a detailed picture of the genetic structuring of crayfish populations is needed, as a remarkable genetic heterogeneity has been detected within *A. pallipes*, *A. italicus* and *A. torrentium* (*e.g.*, present study ; ATTARD and VIANET, 1985 ; LÖRTSCHER *et al.*, 1997, this same volume).

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