CONSERVATION GENETICS OF THE WHITE-CLAWED CRAYFISH *AUSTROPOTAMOBIUS PALLIPES* : THE USEFULNESS OF THE MITOCHONDRIAL DNA MARKER.

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ABSTRACT

The white-clawed crayfish, *Austropotamobius pallipes pallipes*, still has a wide-spread distribution in France, but since the last century, populations have declined because of habitat alteration (due to human disturbance) and have been also eliminated by crayfish plague, for which introduced exotic species are a vector. Action plans for the conservation of *A. pallipes* are urgent and if recovery programmes are to be initiated in France, then it is important to estimate how much genetic variation is partitioned between remaining populations as the species is being currently threatened in all its European distribution. We show how a new molecular marker can be used to study crayfish populations. Mitochondrial DNA (mtDNA) variation in natural populations was examined by restriction fragment length polymorphism analysis in samples taken from fifteen French populations and six European populations representative of three subspecies observed in *A. pallipes* in order to examine the extent of differentiation between populations. Biogeographic considerations about the genetic distances observed between the three subspecies are made. The study reveals a low level of genetic variation among English, Welsh and most of French populations, corresponding to a genetic stock uniformity among *A. pallipes pallipes*. The only two French populations exhibiting a high level of intrapopulational genetic variation are in fact mixed samples : the comparison with results obtained in European populations revealed that the first population was composed of the two subspecies *A. pallipes pallipes* and *A. pallipes italicus* and the second of *A. pallipes italicus* and *A. pallipes lusitanicus*. Results proved that some repopulations, performed in the past from *A. pallipes italicus* and supposed having failed, have been successful and that the French stock did not correspond to the only subspecies *A. pallipes pallipes.* A first analysis of genetic variance observed on a regional scale revealed that there was no genetic structure according to basins and could reflect human-mediated movement of crayfish stocks between these basins. Consequently, mtDNA is an essential marker to

measure genetic diversity between crayfish populations, to map how the subspecies are partitioned in France and what the importance of each is before any planning crayfish conservation strategies of the native crayfish.

Key-words : *Austropotamobius pallipes*, mtDNA, genetic variation, RFLP, population management.

GÉNÉTIQUE ET CONSERVATION DE L'ÉCREVISSE À PATTES BLANCHES *AUSTROPOTAMOBIUS PALLIPES* : UTILITÉ DU MARQUEUR MITOCHONDRIAL.

RÉSUMÉ

L'écrevisse à pattes blanches *Austropotamobius pallipes pallipes* a encore une large répartition en France mais, depuis le siècle dernier, les populations ont été sérieusement réduites à cause de la dégradation des habitats suite à l'activité humaine et ont également été décimées par la peste de l'écrevisse, suite aux introductions d'espèces d'écrevisses exotiques. Si des actions de conservation d'*A. pallipes* sont urgentes à mettre en place, elles ne peuvent se faire sans une estimation préalable de la variabilité génétique des populations chez cette espèce actuellement en voie de disparition. La variabilité de l'ADN mitochondrial (ADNmt) dans les populations naturelles est examinée par polymorphisme de la longueur des fragments de restriction à partir d'échantillons issus de quinze populations françaises et de six populations européennes représentatives de la répartition des trois sous-espèces observées chez *Austropotamobius pallipes*, ceci afin d'examiner l'étendue de la variabilité génétique au sein de l'espèce. Des considérations biogéographiques sont faites à partir des distances génétiques obtenues entre les trois sous-espèces. L'étude révèle un faible niveau de variabilité génétique au sein des populations anglaises, galloises et la plupart des populations françaises, ce qui correspond à une certaine homogénéité génétique du stock parmi les populations d'*A. pallipes pallipes*. En effet, les deux seules populations françaises présentant un haut niveau de variabilité génétique sont en fait des populations mixtes. La comparaison avec les résultats obtenus chez les populations européennes montre que l'une est composée des deux sous-espèces *A. pallipes pallipes* et *A. pallipes italicus* et la deuxième d'*A. pallipes italicus* et *A. pallipes lusitanicus*. Les résultats prouvent que quelques tentatives de repeuplement, faites dans le passé à partir d'individus d'*A. pallipes italicus* et supposées avoir échoué, peuvent avoir réussi et que le stock astacicole français ne doit pas correspondre à la seule sous-espèce *A. pallipes pallipes*. Une première analyse réalisée au niveau régional montre qu'il n'existe pas de structuration génétique entre les bassins hydrographiques, ce qui pourrait traduire l'intervention de l'homme pour des transferts de populations entre bassins. En conséquence, l'ADNmt est un bon marqueur pour mesurer la variabilité génétique au sein des populations d'écrevisses, pour cartographier précisément comment les trois sous-espèces sont réparties en France et quelle est l'importance de chacune avant d'entreprendre toute opération de repeuplement de l'écrevisse autochtone.

Mots-clés : *Austropotamobius pallipes*, ADNmt, variabilité génétique, PLFR, gestion des peuplements.

INTRODUCTION

Austropotamobius pallipes (Lereboullet, 1858), the white-clawed crayfish, has a wide-spread distribution in Europe which stretches from the former Yugoslavia through

Italy, France, Germany, Spain and into Great Britain and Ireland where it reaches the western and northern limits of its range (HOLDICH, 1996). This species is still wide-spread in France, but the number of populations has been considerably reduced over the last few decades (VIGNEUX *et al.*, 1993). Deterioration of water quality (WESTMAN, 1985), habitat destruction, competition from exotic crayfish species (LAURENT, 1988 ; 1997) and, most importantly, the spread of crayfish plague (caused by the fungus *Aphanomyces astaci* Schikora) have had a devastating impact on populations of French native crayfish species. Today, *A. pallipes* has been recently classified as a vulnerable and rare species (GROOMBRIDGE, 1994). It is listed under the EU Habitats Directive and is protected in France. Whilst decisions to protect the habitat and preserve the existing populations have been taken rapidly, it is only recently, when restocking programmes were being planned, that it became clear that these should not be carried out without knowledge of the genetic variation of populations. WAYNE *et al.* (1991), AVISE (1994) and WALDMAN and WIRGIN (1994) have highlighted the importance of obtaining genetic information between remaining populations of an endangered species when recovery programmes are being designed. Unfortunate past results have come from the fact that over the short-term, more species were threatened by extrinsic and environmental factors. Effective long-term conservation planning must incorporate genetic factors. Thus, the viability of species that survive short-term demographic and environmental threats may depend upon the genetic variability they possess and genetic variation can interact with environmental factors in such a way that the two cannot be viewed as independent factors (GILPIN and SOULE, 1986 ; PARTRIDGE and BRUFORD, 1994). If statistically significant genetic differences exist among two or more populations, they may be considered separated stocks and managed as distinct conservation units. Local populations may be adapted to their local environment and introduction of genetically different individuals could adversely alter the gene pool.

In *A. pallipes*, if a recovery programme is to be initiated, then it is important to know how genetic variation is partitioned between remaining listed populations of such an endangered species. In general, very little is known about population genetics in crayfish. Up to 1996, only enzymatic electrophoretic analysis has been performed and has not provided useful markers for crayfish stock identification (NEMETH and TRACEY, 1979 ; BROWN, 1980 ; ALBRECHT and VON HAGEN, 1981 ; ATTARD and PASTEUR, 1984 ; ATTARD and VIANET, 1985 ; BUSACK, 1988, 1989 ; AGERBERG, 1990 ; FEVOLDEN and HESSEN, 1989).

In the last decade, another method of investigating genetic relationships has been developed from the analysis of mitochondrial DNA (mtDNA) using analysis of Restriction Fragment Length Polymorphism (RFLP). Owing to its maternal mode of inheritance and absence of recombination (AVISE *et al.*, 1987 ; WILSON *et al.*, 1985) mtDNA was a favoured genetic system for analysis of population structure. Generally mtDNA offers two important advantages over nuclear genetic markers such as isozymes : the phylogenetic relationships of mtDNA patterns reflect the history of maternal lineages within a population or species ; the ability to detect local differentiation may also be enhanced by the rapid pace of mtDNA evolution which is generally 5 to 10 times faster than of nuclear DNA (AVISE *et al.*, 1987 ; WILSON *et al.*, 1985). The scarcity of papers about mtDNA variation analyses in crustaceans, indeed scarcely any on crayfish, was probably related to the difficulty of extracting total mtDNA for RFLP analysis. Recently, we have outlined a new molecular technique for studying genetic variability in crayfish by adjusting a method for mtDNA extraction and restriction fragment length polymorphism (RFLP) analysis (GRANDJEAN and SOUTY-GROSSET, 1996).

To estimate the suitability of the mtDNA marker in *A. pallipes*, we have investigated the genetic variability in populations sampled on three geographical scales (European, French and French regions). The first objective of this study was to assess levels of genetic variation within the three *A. pallipes* subspecies described by BOTT (1950). According to this author, *A. pallipes pallipes* occurs in Great Britain, Ireland, France, Switzerland and Corsica ; *A. pallipes italicus* in Italy, Dalmatia and Switzerland and *A. pallipes lusitanicus* in the Iberian Peninsula. The three subspecies are considered to be closely morphologically related (LAURENT and SUSCILLON, 1962 ; ALBRECHT, 1980 ; ALMACA, 1987) and consequently, identification problems were raised by management. The second objective was to focus primarily on how much variation occurs in France (national and regional levels). All the results are discussed with regard to biogeographic history and to the conservation of the native species ; first recommendations are given concerning crayfish stock management, seeing that to know to what extent each population could be designated as a separate conservation unit is fundamental in order to decide if management should be implemented at the local or national level. Some results about British populations and other European populations have been extracted from previous papers (GRANDJEAN *et al.*, 1997 a and b) and are compiled with original results obtained in Poitou-Charentes and other French departments (GRANDJEAN, 1997) in order to have an overview by constructing a dendrogram of genetic distances and to discuss the suitability of the mtDNA marker for genetic variability analysis in *A. pallipes.*

MATERIAL AND METHODS

Adult crayfish were collected from six European populations (first part of Table I). Samples were furnished by our European colleagues and represented the three subspecies in *A. pallipes* : *A. p. pallipes* from Great Britain and corresponding to the presumed subspecies present in France ; *A. p. italicus* from Slovenia (Balkans) ; *A. p. lusitanicus* from Spain (Iberian Peninsula).

In the second part of Table I, fifteen French populations were studied : nine were taken from different locations in the region of Poitou-Charentes (France) and six others from locations situated in other French departments. The distribution of these sampled populations according to their hydrographic basins was illustrated in Table I. However, the populations of Pinail and of Puy-de-Dôme were respectively taken from a pond and from a lake.

The number of animals used for genetic study was also reported in Table I. Heart, green glands and testes obtained from fresh animals were used for the mtDNA extraction. The extraction of mtDNA was performed according to the adjusted method by GRANDJEAN and SOUTY-GROSSET (1996).

MtDNA samples were cleaved with six restriction endonucleases : five 6-base cutters (Bgl II, Eco RI, Hind III, Pst I, Xho I) ; one 4-base cutter : Hpa II. Digestions were performed according to the manufacturer's applications (Gibco BRL). The restriction fragments obtained were separated in 1.2 % agarose gels in TE-buffer for 15 h at 30 volts. Gels were stained with SYBR™ Green I (FMC Bioproducts) and visualized with a UV light transluminator. The restriction fragment pattern from each endonuclease was identified by a letter, each individual being characterized by a composite haplotype of six letters.

Table I

Origin of native crayfish *Austropotamobius pallipes* populations sampled in the study. Part 1 : French populations ; Part 2 : European populations. The number of individuals per sample depends upon the state of the population taken from an endangered species and consequently protected (from 15 to 20 if the population is still well established, less than 12 individuals if relic).

Tableau I

Origine des populations de l'écrevisse autochtone *Austropotamobius pallipes* échantillonnées pour cette étude. 1re partie : populations françaises ; 2e partie : populations européennes. Le nombre d'individus prélevés dépend de l'état de la population recensée chez une espèce en danger et donc protégée (de 15 à 20 si la population est encore bien établie, inférieur à 12 individus si la population est de faible densité).

EUROPE

	Basin	Sub-basin	River	Sample
Wales Great Britain		River Wye	River Irfon	10
		River Avon	Broodmead brook	7
			River Sprint	10
			River Wharfe	7
Spain	Province Leon		Ponds	15
Slovenia	Adriatic		River Rizana	20

FRANCE

Department	Basin	Sub-basin	River	Sample
		French populations		
Haute-Vienne	La Vienne		Le Repaire	20
Lozère	Le Rhône	Le Gard	Le Gardon de St-Martin de Lansuscle	7
Orne	La Manche	L'Orne	Le Val Renard	7
Pyrénées-Orientales	La Méditerranée	Le Tech	Le Las Lilas	9
Haut-Rhin	Le Rhin	Le Birsig	La Lucelle	7
Puy-de-Dôme	La Loire	L'Allier	Le Lac Pavin	9
		Poitou - Charentes Region		
Deux-Sèvres	Le Thouet	La Viette	La Martinière	10
	Le Clain	L'Auxances	Le Magot	20
	La Sèvre Niortaise		Le Magnerolle	20
			Le Puits d'Enfer	14
			Le Gatineau	16
Vienne	La Vienne		La Crochatière	20
	Pond		Pinail	5
Charente	La Dronne	La Tude	La Gace	7
	La Charente	La Tardoire	La Fontaine St-Pierre	8

The total proportion of shared fragments (*S*-value) between two individuals was calculated from the following equation (NEI and LI, 1979) :

$$S_{ij} = \frac{2m_{ij}}{m_i + m_j}$$

where m_i and m_j are the numbers of restriction fragments in DNA sequences x and y, respectively, whereas m_{ij} is the number of fragments shared by the two sequences. The number of nucleotide substitutions per site d can be estimated by :

$$\hat{d}_{ij} = \frac{-\ln S_{ij}}{r}$$

where r is the number of bases per restriction site (NEI and LI, 1979). When different kinds of enzymes with different r values are used, the mean number of nucleotide substitutions can be estimated by the formula given by NEI and TAJIMA (1981) :

$$\hat{d}_{ij} = \frac{\sum_k m_k r_k d_{ij}(k)}{\sum_k m_k r_k}$$

where $m_k = \frac{m_{i(k)} + m_{j(k)}}{2}$ and k refers to the kth class of restriction enzymes.

The data were tested for genetic subdivision using analysis of molecular variance (AMOVA) from a program developed by EXCOFFIER *et al.* (1992). This method was used to analyze the genetic structure within and among populations using variance component estimates in a hierarchical analysis. Three hierarchical levels were recognized :

(1) within populations (within each local sampling),

(2) among populations within hydrographic basins,

(3) among hydrographic basins.

The analysis was made from all brooks populations grouping into hydrographic basins.

RESULTS

248 animals were used for mtDNA extraction. All the restriction profiles found in the whole populations are collected in Table II. The size of the mitochondrial genome was estimated by summing the restriction fragment sizes obtained after digestion with the following enzymes Pst I, Eco RI and Xho I (Table II) and was found to be approximately 17 750 ± 580 base pairs.

Populations on an European scale (Tables II and III)

Among the six restriction enzymes, only Xho I produced a monomorphic pattern. Concerning Pst I, there was no restriction site on mtDNA in Spanish and Slovenian crayfish (profile 0) while the molecule was cut in English populations (pattern A). Two patterns were observed for Bgl II : pattern A was shared by English and Slovenian individuals, while pattern B was only related to Spanish crayfish. Three patterns were produced by Eco RI : pattern A was shared by English crayfish while patterns B and C were, respectively, exclusively found in Slovenian and Spanish populations. For Hind III,

patterns A and B were found in English, pattern B being also detected in an individual of the British population from the River Avon (patterns A and B could be explained by the gain or loss of one single restriction site, Table II). Pattern C was shared by Spanish and Slovenian populations. Five patterns were produced by the four-cutter Hpa II (Table II) : patterns A and B were devoted to English populations, the pattern B being specific of one individual from the River Wye. Patterns D and E were found in Slovenian animals and pattern F was characteristic of Spanish crayfish.

Resulting composite haplotypes (Table III) were respectively designed as haplotypes 1, 2 and 3 for English populations, 5 and 6 for Slovenian crayfish and 9 for Spanish sample.

French scale populations (Tables II and III)

Xho I was also monomorphic for all the studied populations. Pst I produced only one pattern A devoted to Le Repaire, Lozère, Haut-Rhin and two individuals taken from Puy-de-Dôme, while there was no restriction site on mtDNA from other crayfish issued from Puy-de-Dôme and those taken from Pyrénées-Orientales. Bgl II produced a pattern A in all the populations except for Pyrénées-Orientales where the three profiles A, B and C were found. Eco RI produced three patterns A, B and D : A is devoted to Le Repaire, Lozère, Haut-Rhin and Orne and two individuals issued from Puy-de-Dôme. Pattern D was only devoted to Pyrénées-Orientales and Puy-de-Dôme, when pattern C was present in one crayfish originating from Pyrénées-Orientales. Four patterns A, G, E and F were found with Hpa II : respectively, A was found in Orne, Le Repaire, Lozère, Haut-Rhin and Puy-de-Dôme ; G in Puy-de-Dôme and Pyrénées-Orientales ; E in Pyrénées-Orientales and F only in one individual taken from Pyrénées-Orientales. Lastly, Hind III produced three patterns A, B and C : A and B were both related to Le Repaire and Lozère, but Orne and Haut-Rhin exhibited only the pattern A. The pattern C was only found in Pyrénées-Orientales and Puy-de-Dôme.

Five haplotypes were consequently defined : the haplotypes 1 and 2 designated Le Repaire and Lozère. Haut-Rhin and Orne have shown no intrapopulational variability (haplotype 1). The latter was also found in a part of Puy-de-Dôme population. Haplotypes 7, 8 and 9 were found in Pyrénées-Orientales and haplotype 8 was also characteristic of Puy-de-Dôme.

Table II

Estimation size in base pairs (bp) of mtDNA restriction fragments for six restriction enzymes. Each letter refers to fragment pattern designation.

Tableau II

Estimation de la taille en paires de base (bp) des fragments de restriction de l'ADNmt pour six enzymes de restriction. Chaque lettre correspond à un profil de restriction.

	HindIII			Hpa II							Eco RI				Bgl II			Xho I	Pst I
	A	B	C	A	B	C	D	E	F	G	A	B	C	D	A	B	C	A	A
	5520	5520	6560	4330	4330	4330	4330	4330	3700	4330	13545	13545	5610	13545	4710	3070	3070	9200x2	8335
	2710	1590	1760	1730	1460	1730	2310	2310	2310	2310	1865	3890	3890	2370	3070	1090	2520		7460
	1425	1425	1425	1460	1250x2	1460	2000	2210	1460	2210x2	935x2	935x2	3000	1505	2520				935x2
	1050	1130	1050	1250	1160x2	1160x2	820	2000	885	820			2730	935x2					
	935	1050	935	1160x2	950	980	760	820	820	760			935x2						
	845	935	845	885	630	885	560	560	760	560									
	615	845	615	630	560	630	485	485	630	485									
	530	615	530	560		560	470		560										
		530							425										
Total	13630	13640	13720	13165	12750	12895	11735	12715	11550	13685	17280	19305	17130	19290	10300	4160	5590	18400	17665

Table III

Number of designated genotypes (1 to 9) and six-letter observed MtDNA haplotypes. Enzymes scored from left to right are : Hind III, Hpa II, Eco RI, Bgl II, Xho I and Pst I.

Tableau III

Nombre de génotypes trouvés (de 1 à 9) et haplotypes correspondants, composés de 6 lettres, les enzymes étant désignés comme suit (de gauche à droite) : Hind III, Hpa II, Eco RI, Bgl II, Xho I et Pst I.

Haplotypes	HindIII	Hpa II	Eco RI	Bgl II	Xho I	Pst I
1	A	A	A	A	A	A
2	B	A	A	A	A	A
3	A	C	A	A	A	A
4	A	B	A	A	A	A
5	C	D	B	A	A	0
6	C	E	B	A	A	0
7	C	E	D	C	A	0
8	C	G	D	A	A	0
9	C	F	C	B	A	0

Populations on a French regional scale (Tables II, III, IV and V)

For four of the six tested restriction enzymes, no difference in the restriction pattern between individuals could be detected : two patterns were observed for Hpa II and Hind III (Table II), but different morphs were separated by a single loss or gain of a restriction site. Hpa II pattern B was only devoted to animals taken from the ponds of Pinail ; three composite haplotypes were detected among the 120 crayfish coming from the nine populations (Table III). No intraspecific variability was apparent in La Martinière, La Gace and La Fontaine Saint-Pierre (haplotype 1), in Le Magot (haplotype 2) and in Pinail (haplotype 4). The third haplotype was only devoted to Pinail. For the other populations showing intraspecific variability by using the restriction enzyme Hind III, details about repartition of haplotypes 1 (7 populations) and 2 (5 populations) in Poitou-Charentes are given in Table IV. Results revealed that the percentages of the two haplotypes 1 and 2 were variable.

For these populations studied on a regional scale, results are expressed in more in detail in Table IV. Analysis of the data for the three hydrographic basins and AMOVA was performed according to EXCOFFIER *et al.* (1992) in order to see the genetic structure per basin and between the basins (Table V). MtDNA nucleotide diversity values within the species ranged from 0.57 to 1.31 %. Estimate of the values is given in Table VI and calculated for the three groups depending upon the three basins : La Vienne, La Charente and La Sèvre Niortaise. The values were significant for the statistical Φsc and Φst. The higher genetic variation was distributed between the populations within basins. There was no geographical structure (Φsc = 64.41 % of total variance) of genetic variability between the different basins, Φct (-0.02) being not significant. The repartition of genetic variability was consequently independent of hydrography Φst, calculated on the whole population, was highly significant (0.624 ; $P < 0.01$).

Table IV

Distribution and percentages of the listed haplotypes in the populations taken from Poitou-Charentes.

Tableau IV

Répartition et fréquence (en pourcentages) des haplotypes recensés dans les populations échantillonnées en Poitou-Charentes.

Department	Basin	Sub-basin	River	Sample	% HAPLOTYPES 1	2	4
Deux-Sèvres	Le Thouet	La Viette	La Martinière	10	100		
	Le Clain	L'Auxances	Le Magot	20		100	
	La Sèvre Niortaise		Le Magnerolle	20	65	35	
			Le Puits d'Enfer	14	86	14	
			Le Gatineau	16	12,5	87,5	
Vienne	La Vienne		La Crochatière	20	75	25	
	Pond		Pinail	5			100
Charente	La Dronne	La Tude	La Gace	7	100		
	La Charente	La Tardoire	La Fontaine St-Pierre	8	100		

Table V

Genetical variance and statistical Φ in the populations taken from Poitou-Charentes, obtained from rivers and gathered according to their hydrographical basin.

Tableau V

Variance génétique et Φ statistiques pour les populations du Poitou-Charentes provenant des cours d'eau et groupées en fonction de leur bassin hydrographique d'origine.

Genetic variance	Φ statistics	
Among hydrographic basins	Φct = - 0.02	P = ns
Among populations within basins	Φsc = 0.631	P < 0.002
Within populations	Φst = 0.624	P < 0.002

Compilation of the whole data (Table VI and Figure 1)

The nucleon diversity percentages were calculated between the nine composite haplotypes and expressed in Table VI : mitochondrial DNA nucleotide diversity values within species ranged from 0.63 % to 12.87 %. There is a lack of genetic differentiation between English and French populations. The most common haplotype (1) was the same for the majority of French populations. However, the geographic differentiation of the mtDNA types between French/English, Spanish and Slovenian populations was evident from the inspection of the geographic locations superimposed on the resulting dendrogram obtained from the NEI's genetic distance (Figure 1). UPGMA revealed three major clusters, one comprising the haplotypes 1, 2, 3 and 4 (*A. p. pallipes*), the second made up of the haplotypes 5, 6, 7 and 8 (*A. p. italicus*) and the third comprising the haplotype 9 (*A. p. lusitanicus*).

Table VI

Nucleon diversity percentages calculated between the different haplotypes found in all the populations (above the diagonal) ; the numbers of different fragments obtained between the haplotypes are expressed below the diagonal.

Tableau VI

Pourcentages de diversité nucléotidique calculés entre les différents haplotypes obtenus dans l'ensemble des populations (au-dessus de la diagonale) ; les nombres de fragments différents obtenus entre les haplotypes sont exprimés en dessous de la diagonale.

Haplotypes	1	2	3	4	5	6	7	8	9
1	—	0.94	0.63	1.39	12.08	11.5	12.08	12.41	12.87
2	3	—	1.59	2.31	12.15	11.48	12.35	12.41	12.87
3	2	5	—	1.81	12.23	11.55	12.07	12.41	12.87
4	4	7	5	—	11.34	10.65	12.65	12.58	12.82
5	19	20	19	21	—	1.26	7.42	2.82	2.87
6	20	21	20	20	3	—	8.57	2.4	1.61
7	27	28	27	27	14	15	—	8.05	9.52
8	24	24	24	25	7	6	17	—	2.05
9	24	24	24	25	7	4	19	5	—

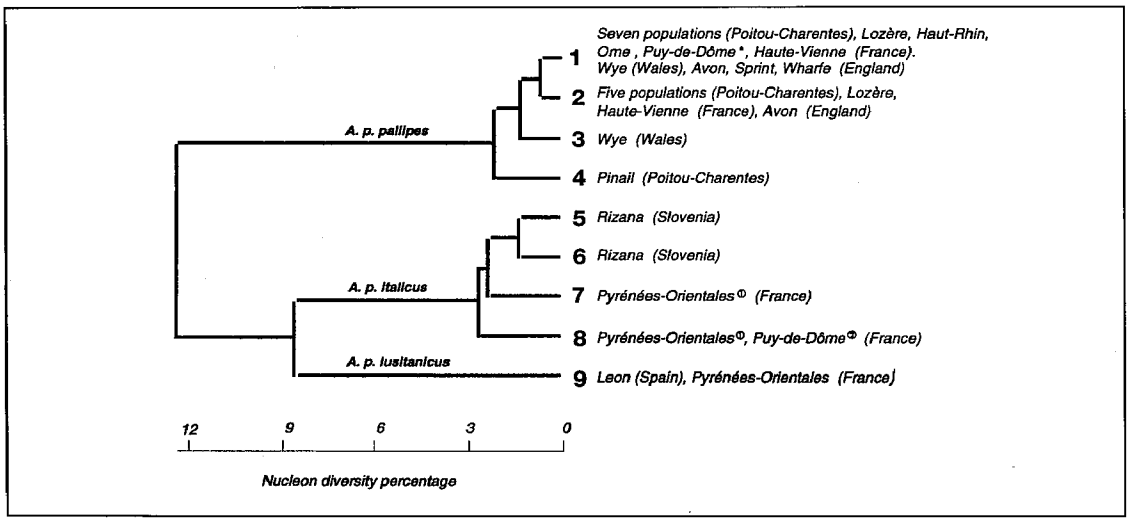

Figure 1

Dendrogram of NEI's genetic distance among several populations of *Austropotamobius pallipes* originating from the distribution area of the species. Genetic relative distances were calculated from Table IV. See Table V for the names of populations taken from Poitou-Charentes and harbouring haplotype 1 and/or haplotype 2.①② : the two populations harbouring two subspecies.

Figure 1

Dendrogramme obtenu à partir des distances génétiques de NEI calculées sur l'ensemble des populations d'*Austropotamobius pallipes* collectées sur l'aire de répartition européenne de l'espèce. Les distances génétiques sont calculées à partir du Tableau IV. Se référer au Tableau V pour la liste précise des populations issues du Poitou-Charentes et hébergeant l'haplotype 1 et/ou l'haplotype 2.①② : les deux populations hébergeant deux sous-espèces.

DISCUSSION

Until recently, no studies have been carried out using mtDNA as a diagnostic marker in population genetics of freshwater crayfish. Our results have allowed an estimate of the size of *A. pallipes* mtDNA to be made (GRANDJEAN and SOUTY-GROSSET, 1996) and this size is that classically found in animals and more particularly in Crustacea Decapoda (see review of OVENDEN, 1990 ; BRASHER *et al.*, 1992 a and b ; BOUCHON *et al.*, 1994 ; SILBERMAN *et al.*, 1994), whereas we have found atypical mtDNA in Isopoda (SOUTY-GROSSET *et al.*, 1992).

Populations on an European scale

Our comparative study of mitochondrial DNA variation between populations has revealed a high level of intraspecific mtDNA sequence diversity in *A. pallipes*. The dendrogram has revealed the existence of three clusters *i.e.* Welsh/English/French, Spanish and Slovenian populations. These three groups could be explained by the presence of the three subspecies described by BOTT (1950). Our results showed that RFLP analysis of mtDNA can provide greater resolution than protein electrophoresis for stock identification because ALBRECHT and VON HAGEN (1981) found no genetic variation in *A. pallipes* populations sampled from different regions of Europe.

The pattern of differentiation of *A. pallipes* European populations was common among freshwater (ALMACA, 1987) and terrestrial species (HEWITT, 1996). Detailed studies of species complexes and wide-spread species have revealed a great deal of geographic subdivision into sibling species, subspecies, races and forms (HEWITT, 1988). According to HEWITT (1996), this subdivision is a genetic consequence of last ice-age. During the ice-age many species which now range across Europe would have had their *refugia* in southern extremities : Iberia, Calabria, Balkans and probably the south of France. Populations in these refuges may well diverge from each other to suit somewhat different habitats and also because of small population sizes responsible for the important phenomenon of genetic drift. Repeated contraction and expansion would accumulate genome differences and adaptations, protected from mixing by hybrid zones, and such a composite mode of speciation could be applied to many organisms (HEWITT, 1996). Consequently, the life history of *A. pallipes* seems to follow the same model : the high genetic differentiation between Spanish/French-British/Slovenian crayfish implies the existence of at least three *refugia* during the last ice-age : the first in Iberian Peninsula, the second in Balkans and the third in the south of France, the north-western Europe being colonized from this latter *refugia* after the last ice period.

UPGMA performed on the haplotypes has revealed the presence of closely related mtDNA clones between English/Welsh and French populations. The fact that mtDNA analysis has failed to reveal a difference between English/Welsh and French populations of *A. p. pallipes* supports the hypothesis that British stock had a post-glacial French origin and that sufficient time has not elapsed to affect genetic homogeneity. ALBRECHT (1982) stated that it was impossible to draw any conclusions about the colonization of the British Isles by *A. p. pallipes*. However, he argued that it was unlikely they could have withstood the permafrost in southern Britain which existed during and immediately after the last ice-age. Thus according to ALBRECHT (1982), *A. p. pallipes* could not have survived the last ice-age in Britain but could have recolonized southern England naturally through post-glacial stream connections with France. According to THIENEMANN (1950), the British Isles were also linked to mainland Europe until 6 000 years ago *via* a land bridge and the rivers of southern England linked up with some European rivers, including the Rhine, Seine, Somme, Authie and Cauche (see HOLDICH and ROGERS, 1997). Another alternative is that the species was introduced by human-mediated movements of crayfish

stocks. THOMAS and INGLE (1971) mentioned that it was a common practice to stock pools and rivers with *A. pallipes* in the past but that there were few mentions of it in the literature before the 17th century. THOMPSON (1843) suggested that *A. pallipes* may have been introduced into Ireland at the beginning of the 19th century. LAURENT (1988) favoured a natural post-glacial colonization of Ireland by *A. pallipes*. The low genetic variability (plus the similarity to the French stock) suggests that crayfish in Britain can be considered as a single stock (GRANDJEAN *et al.*, 1997 a). This may facilitate any restocking programmes which are initiated in response to wide-spread elimination of *A. pallipes* due to outbreaks of crayfish plague. However, if at all possible restocking should be carried out with stock from the nearest surviving populations.

French populations

According to the compilation of data in the dendrogram of NEI's genetic distances, results indicated the presence of the three subspecies in France : *A. p. p.* was present in the populations of Haut-Rhin, Orne, Lozère and all the departments of Poitou-Charentes ; *A. p. i.* was present in Pyrénées-Orientales and Puy-de-Dôme. One individual of Pyrénées-Orientales was identified as *A. p. l.* Consequently, we have revealed the existence of two mixed populations and outlined the role of human activity on the distribution of genetic variation in *A. pallipes*. If the presence of *A. p. l.* on the French side of the Pyrenees could be explained by a natural migration between Spain and France, the presence of *A. p. i.* in the same location was very improbably due to a natural post-glacial colonization from the Italian *refugium* but was without doubt due to human activity. LAURENT and SUSCILLON (1962) pointed out that repopulations with Italian and Spanish white-clawed crayfish have been used for reconstituting a « French stock » after diseases occurring in the 19th century and were responsible for decimating the great majority of French crayfish stock. In Lac Pavin, formed since 6 000 years after a volcanic explosion, RICO (1876) pointed out the first introduction of crayfish (200 individuals of unknown origin). But, seeing that we found two different subspecies in the lake, there were at least two introductions from individuals of different origins.

Populations on a regional scale

Our analysis revealed three haplotypes, the haplotypes 1 and 2 characterizing populations inhabiting streams and haplotype 4 being only devoted to the population found in ponds of Pinail. In Pinail, *A. pallipes* was found in very small ponds (100 m²) with physical and chemical conditions very particular for this species (GRANDJEAN and SOUTY-GROSSET, 1996). In several freshwater decapods, the discontinuous nature of habitats between populations has been a source of genetic divergence (HEDGECOCK *et al.*, 1976 ; FULLER and LESTER, 1980 ; AUSTIN, 1986 ; BUSACK, 1988 ; FEVOLDEN and HESSEN, 1989) although some others have shown low interpopulation divergence (BUSACK, 1988 ; HORWITZ *et al.*, 1990). In our case, this genetic differentiation was due perhaps to a difference in the nature of habitat (GRANDJEAN and SOUTY-GROSSET, 1996). The study of other French populations would also be necessary in order to verify a second hypothesis : it might also be possible that this introduction has been made from individuals originating from another French region.

Concerning the distribution of haplotypes 1 and 2, there was a genetic structure between the populations but there was no structure related to the basins. In the Vienne basin, the genetic composition is variable. The distribution of genetic variability in *A. p. pallipes* has probably been modified by human-mediated movement of crayfish stocks between different basins. Crayfish transplantations which could have altered the natural pattern probably happened early on in man's history (SPITZY, 1979), were a common practice as early in the Middle Ages (LAURENT, 1997) and isolated populations exist as a

result of such activities (HOLDICH and LOWERY, 1988). However, the low genetic diversity found between the two haplotypes obtained from animals coming from streams could lead us to suppose that there still exists a genetic structure on a regional scale, resulting from local restocking.

IMPLICATIONS FOR MANAGEMENT

Much of the advancement in molecular approaches in conservation genetics has developed around better ways of identifying genetic variation so that differences between species and populations can be identified, a task that was not possible with allozymes because rare and endangered species generally are low in allozyme variation (see ATTARD and VIANET, 1985). The analysis of mtDNA marker was undertaken in order to estimate its potentiality in studies of crayfish genetics. Our study of genetic variation has allowed an understanding of both the current and historical evolutionary processes that have generated biodiversity patterns, the preservation of which should be an important component of conservation plans (SMITH *et al.*, 1993). Moreover, from a precautionary point of view, future persistence of populations may depend upon the preservation of specific components of genetic diversity.

On a European scale, our results revealed a high level of genetic variability within the crayfish stock corresponding to the presence of the three subspecies. The high level of interpopulational variability exhibited between Welsh/French, Spanish and Slovenian stocks suggests that within national boundaries except between France and Great Britain, the white-clawed crayfish populations may be considered as separate conservation units. Thus, specific management strategies may be needed in each of these countries. Our results provide strong evidence for a very close genetic relationship among English/Welsh/French populations using the mtDNA method. Therefore, management at national level might be suitable. Clear differences obtained with RFLP patterns indicated that we have a fairly simple diagnostic technique for distinguishing the different stock of the subspecies. In addition to monitoring and protecting extant populations, a primary component of any recovery plan for *A. pallipes* would be an extensive reintroduction programme.

In France, we have shown that the three subspecies were present and even that mixed populations could be constituted. These results are consequently essential for the establishment of management operations : seeing that outbreeding could alter the gene pool, concrete recommendations could be given taking account that the mixing populations should be avoided in the case of restocking operations. Our results have also raised the absolute neccessity to exhaustively study a great number of French populations in order to know exactly the respective extent of the three subspecies in France, before trying to manage the protection of the autochtonous subspecies *A. p. pallipes*.

At a regional scale, the very low level of genetic variation within *A. p. pallipes* subspecies led to the conclusion that the populations probably represent a single stock. However, it is necessary to further investigate mtDNA diversity in more populations taken from other regions in order to resolve the question if there still exists a genetic structure on a regional scale as a consequence of human operations.

Results showed that mtDNA could be a reliable marker for characterizing the three subspecies but further work is needed to appreciate the suitability of this marker to reveal genetic variability within *A. p. pallipes*. In the case of genetic homogeneity within *A. p. pallipes*, other molecular markers will have to be investigated : more sensitive molecular tools (such as analyses of microsatellites) may permit more wide-spread applications of molecular approaches to the problems of *A. p. pallipes* conservation.

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