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APPLIED ISSUES

Population genetic structure of the endangered crayfish *Austropotamobius pallipes* in France based on microsatellite variation: biogeographical inferences and conservation implications

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SUMMARY

1. One important goal in conservation biology is to characterise evolutionary lineages within endangered species before management decisions are taken. Here, we assess population differentiation in the freshwater crayfish *Austropotamobius pallipes*, an endangered species endemic to western Europe and provide valuable information for the conservation of French populations.
2. Analysis of five microsatellite loci in 44 populations revealed very different within population levels of genetic diversity ($0.000 < H_0 < 0.564$). Two groups, corresponding to northern and southern French populations, showed a high degree of genetic differentiation in both allele frequencies and allele sizes. Comparison of these results with previous studies of *A. pallipes* strongly suggests that the divergence between northern and southern populations could have occurred during the last glaciation period of the Pleistocene from one Atlantic and one Mediterranean refuge.
3. Evidence for genetic admixture between these two lineages was revealed by correspondence analyses in southern populations, probably as the result of artificial translocations.
4. French populations appeared significantly differentiated among the different river drainages and were highly structured within rivers. The impact of population size, population bottlenecks and founder events on the population genetic differentiation are discussed.
5. Based on these results, we propose the designation of two evolutionarily significant units for *A. pallipes* in France. Our data also support the maintenance of separate demographic management strategies for crayfish inhabiting different river systems. However, genetic analyses will have to be combined with demographic and ecological data for sustainable conservation programmes.

Keywords: *Austropotamobius pallipes*, conservation genetics, evolutionarily significant unit, microsatellites, secondary contact

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Introduction

Maintaining the evolutionary potential of endangered species is a fundamental goal in conservation biology. In 1986, Ryder developed the concept of the evolutionarily significant unit (ESU), characterised by

populations harbouring multiple concordant characters based on ecological, ethological, biogeographical and morphological data (Ryder, 1986). Since then, various definitions have been formulated and widely discussed (reviewed in Fraser & Bernatchez, 2001). The most significant and recent disagreement focused on the importance of including ecological data when defining ESUs. In fact, Crandall *et al.* (2000) argued that the exclusive use of neutral genetic markers did not place enough emphasis on the potential of species to maximise evolutionary success through the maintenance of adaptive diversity. However, in an effort to unify the various concepts of ESU, Fraser & Bernatchez (2001) concluded that each approach had its strengths and weaknesses under different circumstances and thus could be used alone or in combination depending on the situation.

The white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet 1858), is endemic to Western Europe. This species is experiencing a drastic decline in natural populations that has been attributed to habitat loss, water pollution and the effects of competition and diseases from introduced crayfish species (Changeux, 2003). *Austropotamobius pallipes* has been listed in the red book of endangered species since 1983 (Baillie & Groombridge, 1996) and listed under Annex 2 of the EU Habitats Directive as a species requiring special conservation measures. Because French *A. pallipes* populations had not received much detailed study, the decision was made to employ a molecular genetic approach in order to evaluate the distribution of the genetic variation in the French stock. Such an approach is particularly appropriate for species suffering a rapid population decline because it provides important information for management strategies if such actions need to be employed (Hedrick, 2000). Furthermore, according to Moritz (1994), the accumulation of genetic differences through reproductive isolation is a critical factor to consider in defining evolutionary lineages for conservation. He therefore proposed to define ESUs as groups of populations that are reciprocally monophyletic for mitochondrial DNA alleles and show significant divergence of allele frequencies at nuclear loci.

Mitochondrial DNA variation in French populations of *A. pallipes* has been analysed by Grandjean & Souty-Grosset (2000). Using restriction fragment length polymorphism (RFLP) analysis in 21 populations, they identified two lineages of mtDNA haplotypes showing reciprocal monophily and separating northern from

southern French populations. According to the definition of Moritz (1994), polymorphism at nuclear loci has to be investigated in order to support the hypothesis of two ESUs in *A. pallipes* in France. The random amplified polymorphic DNA (RAPD) method was used in a study of 17 French populations (Gouin *et al.*, 2001). As described with mtDNA by Grandjean & Souty-Grosset (2000), RAPDs revealed significant genetic differentiation between northern and southern populations. In light of this result, the hypothesis of deep divergence between these two groups of populations, probably resulting from an ancient separation during the glaciations of the Pleistocene, was proposed. However, four populations sampled in eastern France appeared genetically differentiated from the northern and southern ones, suggesting the possible existence of a third refuge area during the Pleistocene and therefore of a potential third ESU. In this context and due to the lack of statistical power of RAPD analyses, the use of additional nuclear markers was needed to clarify the genetic structure between French populations of *A. pallipes*. Microsatellite markers have recently been isolated for this species (Gouin, Grandjean & Souty-Grosset, 2000; Gouin *et al.*, 2002). These codominant genetic markers are highly polymorphic, more informative than dominant markers such as RAPDs and now constitute the most commonly used method to assess population genetic structure on a fine scale. Moreover, the two studies described above were based on a limited number of populations and important French river basins were not represented (the Adour and the Seine) or poorly represented (only one population from the Garonne). Therefore, a more extensive sampling effort was needed in order to get a better picture of the genetic differentiation in the French stock.

The objective of this study was to examine the genetic variation at five microsatellite loci within French populations of *A. pallipes* and to analyse its distribution in a large sample representative of the distribution of the species in France. Our main focus was to discuss the relevance of these results for the conservation of the white-clawed crayfish.

Methods

Sample collection

Austropotamobius pallipes is still widespread in France, but it is now confined largely to headwater river

systems (Changeux, 2003). Most of populations are located in the southern part of France, the northern part harbouring only scattered populations. In order to cover most of the range of *A. pallipes*, a total of 43 populations were sampled in the main French river basins between 1999 and 2001 (Fig. 1; Table 1). One additional population was sampled in Germany (Fribourg) close to the French border. One pereiopod of the fourth pair was taken from each individual and stored in pure ethanol until DNA extraction. The number of individuals per sample is listed in Table 1.

Microsatellite analysis

Gouin *et al.* (2000) identified four polymorphic microsatellites in *A. pallipes*: *Ap2*, *Ap3*, *Ap5* and *Ap6*. More recently, Gouin *et al.* (2002) characterised an additional locus *Ap7* in a French population and designed

a new forward primer for *Ap5* to facilitate its analysis. These five microsatellites were used in the present study to assess genetic variation in *A. pallipes*. All experimental procedures for DNA extraction, microsatellite amplification and genotyping were identical to those described in Gouin *et al.* (2002).

Locus *Ap5* generated alleles with complex patterns of amplification having many stutter bands. For this reason, each individual was scored twice independently at this locus, and reamplified in case of discordance. Individuals were then rescored and any ambiguous genotype was blanked.

Statistical analysis

The average number of alleles per locus (A), the observed heterozygosity (H_O) and the expected heterozygosity (H_E) were computed with GENETIX 4.05

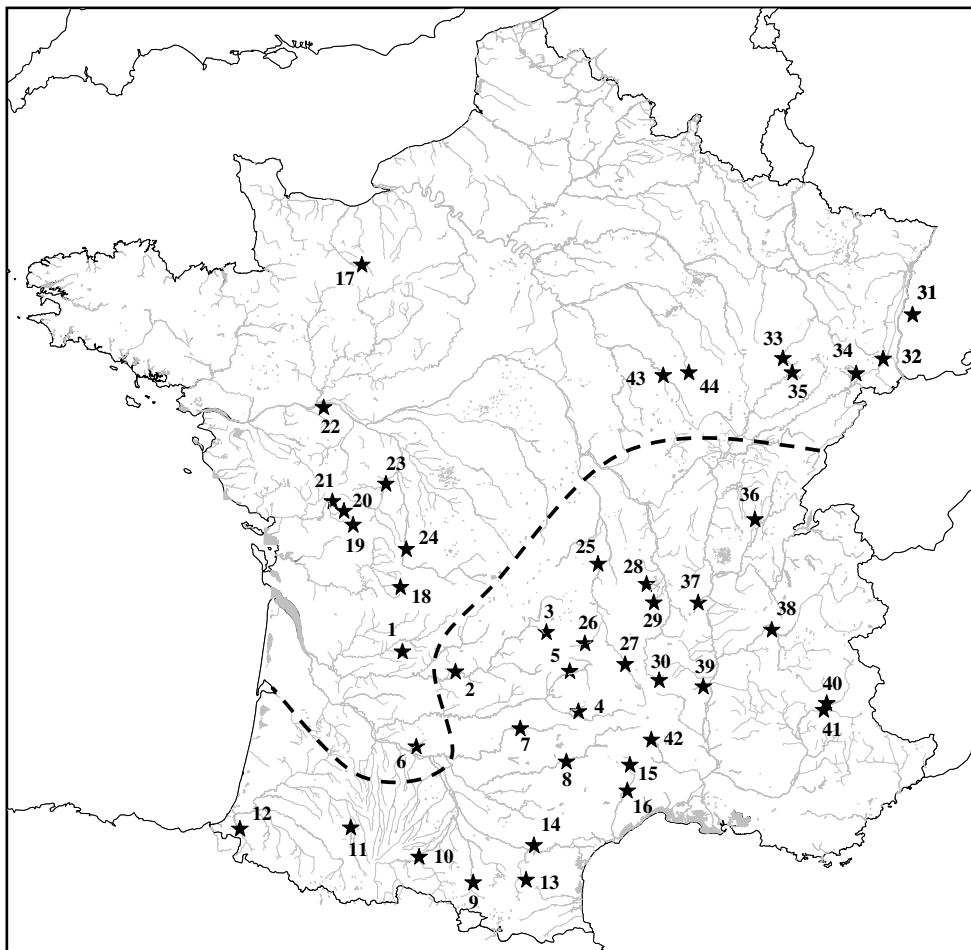


Fig. 1 Geographical position of the 44 French populations of *A. pallipes* sampled for this study. The dashed line represents the boundary between northern (NJ group I) and southern (NJ group II) populations.

Table 1 Description of the populations sampled for this study (name, river basin, code used in the analyses, sample size = *N*) and summary statistics for the five microsatellites analysed

Population	Major drainage	Code	<i>N</i>	<i>A</i>	<i>H_O</i>	<i>H_E</i>
Le Serre	Garonne	GAR-1	20	2.0	0.223	0.207
Saint Romain		GAR-2	12	3.0	0.583	0.564
Rau le Mardaret		GAR-3	11	1.4	0.058	0.056
Boralde d'Aubrac		GAR-4	20	1.4	0.120	0.117
Pierrefont		GAR-5	10	1.6	0.147	0.199
Brezegues		GAR-6	11	2.0	0.365	0.335
Combe Nègre		GAR-7	20	1.2	0.100	0.100
Lavandou		GAR-8	19	1.4	0.044	0.075
Artix* [†]		GAR-9	14	3.0	0.400	0.468
Belbèze		GAR-10	5	2.4	0.400	0.347
Louet	Adour	ADO-11	19	2.6	0.442	0.456
Lys		ADO-12	18	2.4	0.287	0.296
Laurio*	Aude	AUD-13	12	1.0	0.000	0.000
Serremijannes*		AUD-14	10	1.2	0.022	0.022
Le Garrel	Hérault	HER-15	7	1.4	0.040	0.057
Combe Bonne*		HER-16	18	1.2	0.023	0.023
Val Renard* [†]	Orne	ORN-17	12	1.2	0.073	0.104
Fontaine St Pierre* [†]		CHA-18	11	1.6	0.200	0.205
Renardières	Sèvre Niortaise	SEV-19	19	2.0	0.284	0.278
Marcusson		SEV-20	24	1.8	0.275	0.276
Breuil	Loire	SEV-21	20	2.6	0.334	0.349
Brocard		LOI-22	30	2.4	0.329	0.357
Macre		LOI-23	22	2.0	0.313	0.336
Crochatière* [†]		LOI-24	19	1.8	0.282	0.248
Fourneaux		LOI-25	7	1.4	0.190	0.159
Alagnon		LOI-26	10	1.6	0.182	0.155
Besque [†]		LOI-27	20	1.6	0.133	0.172
Périchon*		LOI-28	14	1.8	0.157	0.200
Curraize*		LOI-29	14	2.0	0.222	0.265
Mejeanne*		LOI-30	20	1.6	0.170	0.170
Fribourg (DE) [†]	Rhin	RHI-31	9	1.6	0.208	0.207
La Lucelle* [†]		RHI-32	10	1.8	0.340	0.332
Ru des menottes	Rhône	RHO-33	16	2.2	0.400	0.412
Madeleine [†]		RHO-34	12	2.0	0.317	0.299
Le Rupt [†]		RHO-35	24	1.8	0.258	0.272
Ségnac [†]		RHO-36	16	2.8	0.262	0.345
Mornante		RHO-37	10	1.8	0.180	0.181
Isère [†]		RHO-38	15	2.2	0.188	0.216
Mezayon		RHO-39	18	2.6	0.300	0.289
Serre-Ponçon		RHO-40	20	1.6	0.183	0.208
Turrelet		RHO-41	20	2.0	0.270	0.283
Gardon St Martin* [†]		RHO-42	8	1.4	0.111	0.173
Ru du Verpan	Seine	SEI-43	19	2.0	0.233	0.225
Seine		SEI-44	10	1.6	0.180	0.289

Significant departures from Hardy–Weinberg equilibrium prior to the sequential Bonferroni corrections are reported in bold.

*Populations analysed by RFLP mtDNA (Grandjean & Souty-Grosset, 2000).

[†]Populations analysed by RAPD (Gouin *et al.*, 2001).

A, mean number of alleles per locus; *H_O*, observed heterozygosity; *H_E*, expected heterozygosity).

(Belkhir *et al.*, 1996–2004). Unbiased expected heterozygosity (Nei, 1978) was retained because of the small sample size of some populations. Deviations from Hardy–Weinberg equilibrium were tested using the

exact probability test of Guo & Thompson (1992) available in GENEPOP 3.2a (Raymond & Rousset, 1995). Significance levels were calculated at each locus for each population and over all loci for each popula-

tion. Genotypic linkage disequilibrium between each pair of loci was estimated by Fisher's exact tests with GENEPOP 3.2a software. Both tests for deviations from Hardy–Weinberg equilibrium and for linkage disequilibrium used a Markov chain (1000 dememorisation steps, 100 batches, 1000 iterations/batch).

The PHYLIP package (Felsenstein, 1993) was used to calculate Cavalli-Sforza & Edwards (1967) chord distances between populations and to perform a neighbour-joining (NJ) cluster analysis. Statistical support for the branching pattern was obtained with 2000 bootstrap procedures. Significant difference in allelic frequencies was determined by testing the null hypothesis of homogeneity in allelic distribution between the clusters identified in the NJ tree using a Fisher's exact test. This test was performed with GENEPOP 3.2a using the Markov chain method (1000 dememorisation steps, 100 batches, 1000 iterations/batch). In order to test if stepwise-like mutations at microsatellite loci contributed significantly to the genetic differentiation between clusters, we implemented a test based on a randomisation procedure of allele sizes proposed by Hardy *et al.* (2003) using the software SPAGeDI 1.1 (Hardy & Vekemans, 2002). A significant difference indicates that allele-size based statistics (R_{ST}) perform better than identity-based ones (F_{ST}). This test can also provide valuable insights into the main causes of population differentiation (i.e. mutation versus drift) by comparing F_{ST} and R_{ST} values. F_{ST} and R_{ST} are expected to be of the same magnitude when differentiation is only caused by drift, whereas R_{ST} is expected to be larger than F_{ST} under the contribution of stepwise-like mutations (see Lugon-Moulin *et al.*, 1999; Hardy *et al.*, 2003). We also performed this test between populations within clusters to determine the proper statistic for assessing their genetic differentiation. Significance was estimated using 1000 randomisation procedures. Based on the results of these tests, genetic differentiation between populations was estimated using pairwise F_{ST} estimates according to Weir & Cockerham (1984) and pair-wise ρ_{ST} estimates, an unbiased estimator of Slatkin's R_{ST} , according to Michalakis & Excoffier (1996). Pair-wise F_{ST} and ρ_{ST} values were calculated using ARLEQUIN 2.0 software (Schneider, Roessli & Excoffier, 2000). Significance was estimated using 10 000 permutations. For each cluster, a hierarchical analysis of molecular variance (AMOVA, Excoffier,

Smouse & Quattro, 1992) was used to assess the partitioning of microsatellite variation in allelic frequencies (F_{ST}) among populations between and within river basins. AMOVA calculations were performed using ARLEQUIN 2.0 software and the significance of the variance components was tested using 10 000 permutations. We also investigated the possibility of secondary contacts between *A. pallipes* lineages because the NJ analysis revealed the presence of different lineages in the same river drainage. Because of the limited number of microsatellite markers available and because of their relatively low polymorphism, we used correspondence analysis (CA) rather than other methods that are more computationally intensive and that require more markers. CA was performed on the matrix of allele counts per sample at the population and the individual levels using GENETIX 4.05 software. This qualitative approach is useful for analysing the homogeneity of a given sample, and it was proven very efficient in the study of hybrid zones and in the identification of admixed populations (Daguin, Bonhomme & Borsa, 2001; Aurelle, Cattaneo-Berrebi & Berrebi, 2002; Bierne *et al.*, 2003).

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

Results

The microsatellite markers showed a relatively low level of within population polymorphism, but exhibited important variations between populations. Allelic frequencies in the 44 populations of *A. pallipes* are reported in the Appendix. The number of alleles per locus ranged from 2 for the locus *Ap6* to 15 for the locus *Ap7* (Table 2). The mean allelic diversity per locus varied between 1.0 in the population of Laurio to 3.0 in the populations of Artix and Saint Romain, with an average of 1.9 over all populations (Table 1). The proportion of expected heterozygous individuals per population ranged between 0.000 and 0.564 (Table 1). The mean expected heterozygosity in the French populations of *A. pallipes* was 0.235. After sequential Bonferroni correction for multiple tests, no significant departure from Hardy–Weinberg equilibrium was observed in each population, both at each

Table 2 Total number of alleles per locus (*A*), observed (*H_O*) and expected (*H_E*) heterozygosity, deviation from Hardy–Weinberg equilibrium (*F_{IS}*) for each locus in each population

Locus	<i>Ap2</i>				<i>Ap3</i>				<i>Ap5</i>				<i>Ap6</i>				<i>Ap7</i>			
	<i>A</i>	<i>F_{IS}</i>	<i>H_O</i>	<i>H_E</i>	<i>A</i>	<i>F_{IS}</i>	<i>H_O</i>	<i>H_E</i>	<i>A</i>	<i>F_{IS}</i>	<i>H_O</i>	<i>H_E</i>	<i>A</i>	<i>F_{IS}</i>	<i>H_O</i>	<i>H_E</i>	<i>A</i>	<i>F_{IS}</i>	<i>H_O</i>	<i>H_E</i>
GAR-1	1	–	–	–	1	–	–	–	3	+0.05	0.35	0.37	2	–0.17	0.50	0.43	3	–0.08	0.26	0.24
GAR-2	3	–0.11	0.66	0.60	2	+0.20	0.42	0.52	4	+0.01	0.67	0.67	2	–0.16	0.33	0.29	4	–0.14	0.83	0.73
GAR-3	1	–	–	–	2	–	0.09	0.09	2	–0.06	0.20	0.19	1	–	–	–	1	–	–	–
GAR-4	1	–	–	–	2	+0.02	0.35	0.36	2	–0.11	0.25	0.22	1	–	–	–	1	–	–	–
GAR-5	1	–	–	–	1	–	–	–	2	+0.23	0.33	0.42	1	–	–	–	3	+0.31	0.40	0.57
GAR-6	2	–0.23	0.64	0.52	1	–	–	–	3	+0.04	0.54	0.57	2	–0.13	0.54	0.48	2	–	0.10	0.10
GAR-7	1	–	–	–	1	–	–	–	2	–	0.05	0.05	1	–	–	–	1	–	–	–
GAR-8	1	–	–	–	2	–	0.05	0.05	1	–	–	–	1	–	–	–	2	+0.49	0.16	0.32
GAR-9	3	+0.21	0.43	0.54	3	+0.20	0.36	0.44	2	–0.20	0.38	0.32	2	+0.30	0.21	0.30	4	+0.09	0.61	0.73
GAR-10	2	–	0.20	0.20	2	–	0.20	0.20	2	–0.14	0.40	0.35	2	–	0.20	0.20	5	–0.33	1.00	0.78
ADO-11	3	+0.06	0.53	0.56	2	+0.39	0.21	0.34	2	–0.06	0.42	0.40	2	+0.08	0.31	0.34	4	–0.15	0.74	0.64
ADO-12	3	+0.18	0.39	0.47	2	–0.13	0.28	0.25	3	+0.05	0.44	0.46	2	–	0.05	0.05	2	–0.13	0.28	0.25
AUD-13	1	–	–	–	1	–	–	–	1	–	–	–	1	–	–	–	1	–	–	–
AUD-14	1	–	–	–	1	–	–	–	2	–	0.11	0.11	1	–	–	–	1	–	–	–
HER-15	1	–	–	–	2	–	0.14	0.14	1	–	–	–	1	–	–	–	2	–	0.14	0.14
HER-16	1	–	–	–	1	–	–	–	2	–0.03	0.12	0.11	1	–	–	–	1	–	–	–
ORN-17	1	–	–	–	1	–	–	–	1	–	–	–	1	–	–	–	2	+0.31	0.36	0.52
CHA-18	2	–	0.09	0.09	1	–	–	–	2	+0.13	0.36	0.41	2	–0.05	0.54	0.52	1	–	–	–
SEV-19	2	–0.03	0.10	0.10	2	–0.06	0.16	0.15	2	–0.02	0.47	0.46	2	+0.53	0.42	0.44	2	–0.12	0.26	0.23
SEV-20	2	+0.16	0.42	0.50	2	–	0.04	0.04	2	+0.01	0.42	0.42	2	–0.19	0.50	0.42	1	–	–	–
SEV-21	2	–0.19	0.35	0.30	2	–0.06	0.15	0.14	4	+0.15	0.53	0.61	2	+0.24	0.20	0.26	3	–0.04	0.44	0.43
LOI-22	2	–0.12	0.57	0.51	1	–	–	–	4	+0.20	0.41	0.51	2	+0.30	0.33	0.47	3	–0.15	0.33	0.29
LOI-23	2	+0.18	0.36	0.44	3	–0.04	0.64	0.61	2	+0.13	0.43	0.49	1	–	–	–	2	–0.05	0.14	0.13
LOI-24	2	–0.29	0.47	0.37	1	–	–	–	3	+0.07	0.37	0.40	2	–0.22	0.56	0.46	1	–	–	–
LOI-25	1	–	–	–	1	–	–	–	2	–0.33	0.67	0.53	2	–0.09	0.28	0.26	1	–	–	–
LOI-26	1	–	–	–	2	–0.29	0.50	0.39	2	–0.12	0.30	0.27	1	–	–	–	2	–	0.11	0.11
LOI-27	1	–	–	–	2	+0.31	0.30	0.43	2	+0.20	0.27	0.33	1	–	–	–	2	–0.03	0.10	0.10
LOI-28	1	–	–	–	2	–	0.07	0.07	2	+0.30	0.21	0.30	1	–	–	–	3	+0.21	0.50	0.63
LOI-29	1	–	–	–	2	–0.08	0.21	0.20	2	+0.25	0.36	0.45	1	–	–	–	4	+0.21	0.54	0.67
LOI-30	1	–	–	–	2	–0.10	0.55	0.50	2	+0.16	0.25	0.30	1	–	–	–	2	–	0.05	0.05
RHI-31	2	–0.23	0.44	0.37	1	–	–	–	2	+0.19	0.37	0.46	2	–0.07	0.22	0.21	1	–	–	–
RHI-32	2	–0.38	0.60	0.44	1	–	–	–	2	+0.22	0.40	0.50	2	+0.04	0.50	0.52	2	–0.06	0.20	0.19
RHO-33	2	–0.22	0.56	0.47	3	+0.24	0.37	0.49	3	+0.22	0.50	0.64	2	–0.21	0.56	0.46	1	–	–	–
RHO-34	3	–0.27	0.83	0.66	1	–	–	–	2	+0.20	0.42	0.52	2	–0.10	0.25	0.23	2	–	0.08	0.08
RHO-35	2	+0.33	0.21	0.31	1	–	–	–	3	+0.19	0.46	0.56	2	–0.29	0.62	0.49	1	–	–	–
RHO-36	1	–	–	–	2	+0.65	0.06	0.17	4	+0.16	0.50	0.59	2	–0.11	0.25	0.22	5	+0.32	0.50	0.73
RHO-37	1	–	–	–	2	–0.12	0.30	0.27	2	+0.10	0.40	0.44	1	–	–	–	3	–0.03	0.20	0.19
RHO-38	1	–	–	–	2	–0.04	0.13	0.13	3	–0.07	0.27	0.25	1	–	–	–	4	+0.24	0.53	0.70
RHO-39	1	–	–	–	3	–0.04	0.17	0.16	2	–0.03	0.50	0.48	1	–	–	–	6	–0.04	0.83	0.80
RHO-40	1	–	–	–	2	–0.15	0.30	0.26	2	+0.30	0.26	0.37	1	–	–	–	2	+0.15	0.35	0.41
RHO-41	1	–	–	–	2	–0.04	0.40	0.38	3	+0.22	0.35	0.45	1	–	–	–	3	–0.03	0.60	0.58
RHO-42	1	–	–	–	2	+0.63	0.12	0.32	2	+0.22	0.43	0.54	1	–	–	–	1	–	–	–
SEI-43	2	–0.12	0.47	0.42	2	–0.03	0.10	0.10	2	+0.08	0.31	0.34	2	–0.06	0.16	0.15	2	–0.03	0.11	0.11
SEI-44	2	+0.25	0.40	0.53	2	+0.56	0.20	0.44	2	+0.39	0.30	0.48	1	–	–	–	1	–	–	–

Significant departures from Hardy–Weinberg equilibrium prior to the sequential Bonferroni corrections are reported in bold.

locus (Table 2) and over all loci (Table 1). It is also important to note that despite the scoring difficulties mentioned previously, no deviation from Hardy–Weinberg equilibrium was observed at locus *Ap5*

over all populations, suggesting that genotypes were correctly scored for most or all individuals. No significant linkage disequilibrium was revealed between any of the locus pairs (data not shown).

The NJ dendrogram based on the Cavalli-Sforza & Edwards (1967) chord distance revealed that the genetic differentiation between populations was gradual from south to north, but with two genetically differentiated groups corresponding to northern (group I) and southern (group II) French populations (Fig. 2). These two entities displayed significant differences in allele frequencies at the five microsatellite

loci ($P < 0.001$). Allele frequencies for each group of populations are shown in Fig. 3. The test based on allele sizes permutations revealed that stepwise-like mutations contributed significantly to the genetic differentiation between northern French and southern French populations ($P = 0.028$), indicating that R -statistics would be more suitable than F -statistics to assess genetic differentiation between these line-

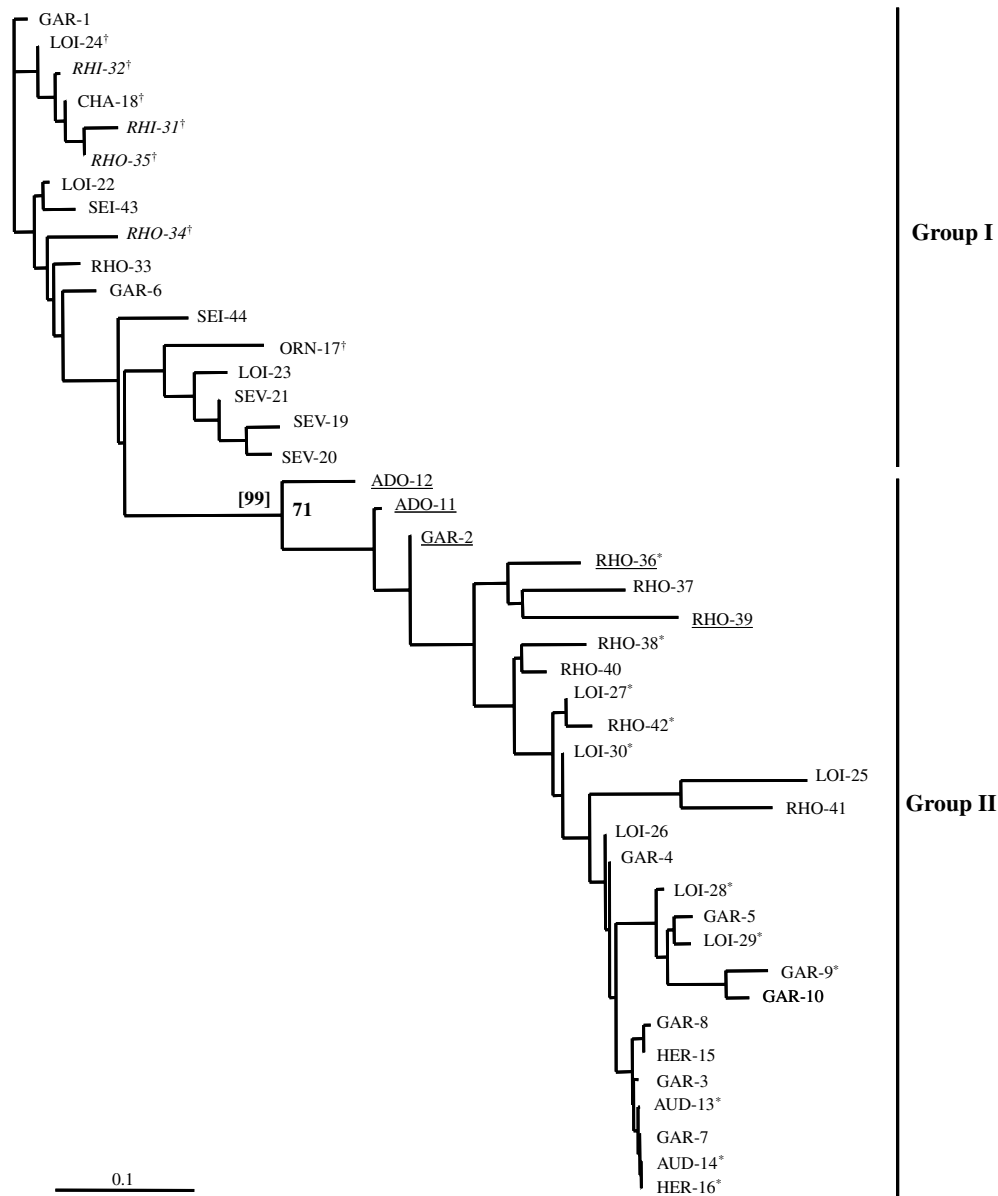


Fig. 2 Neighbour-joining dendrogram based on Cavalli-Sforza & Edwards (1967) chord distances between French populations. Bootstrap values (2000 replicates) are shown if $>55\%$. The value reported in square brackets corresponds to the bootstrap value obtained after removing the five admixed populations (underlined). We have indicated the populations previously assigned to a northern (†) or a southern (*) French lineage by Grandjean & Souty-Grosset (2000) and Gouin *et al.* (2001). Populations in italics correspond to the north-eastern lineage proposed by Gouin *et al.* (2001).

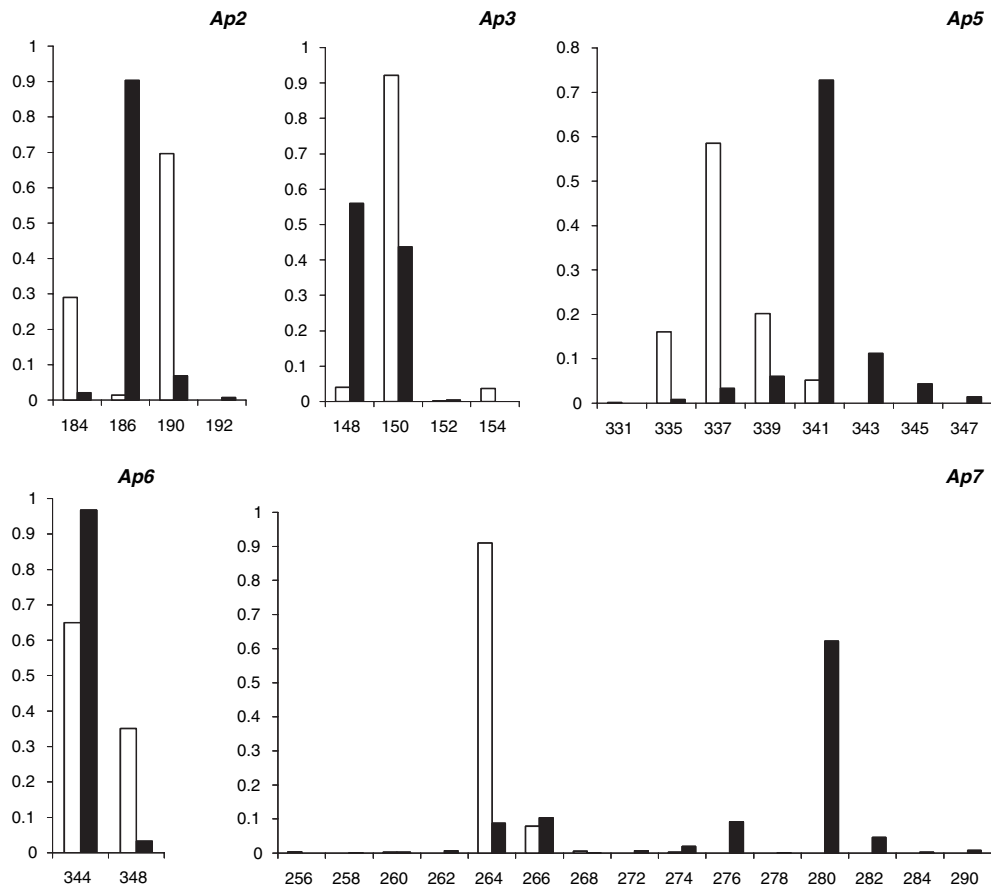


Fig. 3 Distribution of allele frequencies in the two lineages of *A. pallipes* for each microsatellite locus (group I in white; group II in black).

ages. No significant effect of stepwise-like mutations was detected between populations within each of the groups ($P > 0.05$), illustrating that genetic drift was the main force responsible for the genetic differentiation observed between populations within groups and that F -statistics would be more appropriate for measuring population structure within each lineage.

Pair-wise F_{ST} and ρ_{ST} revealed that most of the French *A. pallipes* populations were highly genetically differentiated, except north-eastern populations (RHI-31/32, RHO-33/34/35) and a group of southern populations (GAR-3/4/7/8, AUD-13/14, HER-15/16; Table 3). A few populations with very low sample size (GAR-10, LOI-25, RHI-32, RHO-42) displayed high F_{ST} values that were not significant, and will require additional samples. As described in Table 4, genetic differentiation appeared more pronounced between populations sampled in southern France ($F_{ST} = 0.461$; $P < 0.001$) compared with populations

sampled in northern France ($F_{ST} = 0.226$; $P < 0.001$). AMOVA also revealed that northern and southern French populations of *A. pallipes* were significantly differentiated between the different major drainages (group I: $F_{CT} = 0.08$; $P = 0.007$; group II: $F_{CT} = 0.265$; $P < 0.001$). About 15% of the total genetic variance was attributable to differentiation between populations within river basins in northern populations and about 20% in southern populations, indicating that they were genetically structured. However, it must be noted that there was more variation within river basins than between river basins in northern populations and almost as much variation within as between basins in southern populations.

The CA performed on *A. pallipes* populations clearly separated populations from NJ group I and II along the first axis, with an inertia of 36.6% (Fig. 4). However, a group of five southern populations from NJ group II, Saint Romain (GAR-2), Louet (ADO-11),

Variance components	d.f.	% Total variance	F-statistics	P-value
Northern French populations (group I)				
Among river basins	7	8.22	$F_{CT} = 0.082$	0.007
Among populations within river basins	9	14.38	$F_{SC} = 0.157$	<0.001
Within populations	559	77.40	$F_{ST} = 0.226$	<0.001
Southern French populations (group II)				
Among river basins	5	26.52	$F_{CT} = 0.265$	<0.001
Among populations within river basins	21	19.63	$F_{SC} = 0.267$	<0.001
Within populations	747	53.85	$F_{ST} = 0.461$	<0.001

Table 4 Hierarchical partitioning of genetic variance (AMOVA) at microsatellite loci among populations between and within river basins in northern and southern French populations

Lys (ADO-12), Sélignac (RHO-36) and Mezayon (RHO-39), appeared in fact to have an intermediate genetic composition between populations of the two lineages. It is important to note that the bootstrap value between the two NJ groups reaches 99% when these five populations are removed from the analysis (Fig. 2). The CA implemented on the individual genotypes confirmed the heterogeneous composition of the populations described above, with individuals distributed between the two groups (Fig. 5). One sample from the population of Saint Romain (GAR-2) was clearly plotted among individuals from NJ group I along the first CA axis. Populations of Louet (ADO-11) and Lys (ADO-12) also showed specimens with a genetic composition close to that of individuals from NJ group I. Most of the individuals from Lys were distributed between the two groups.

Discussion

The primary objective of this study was to quantify the extent of microsatellite variation in the French populations of *A. pallipes*. Our results revealed only a moderate level of polymorphism despite an exhaustive sampling regime. This finding is not surprising as freshwater crayfish are known to display a low genetic variability (Fetzner & Crandall, 2001). However, with an average level of heterozygosity of 0.235, the polymorphism revealed with the microsatellites was much higher than the polymorphism previously recorded from allozymes and RAPDs (Attard & Vianet, 1985; Gouin *et al.*, 2001). This variation was sufficient to provide a clear picture of the genetic differentiation among French populations, between and even within river basins.

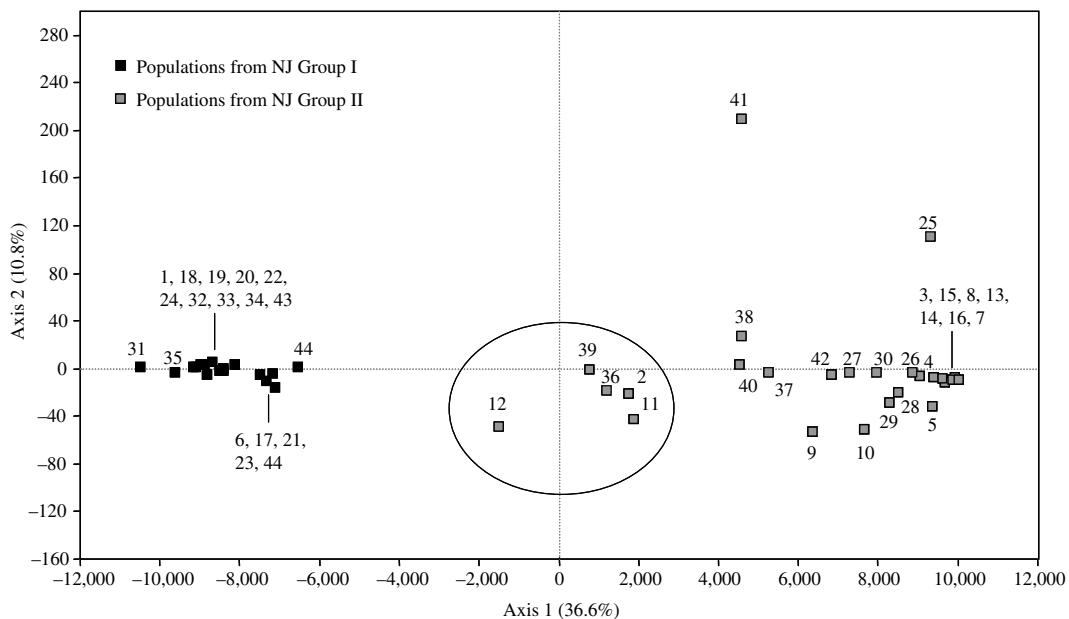


Fig. 4 Correspondence analysis: projection of *A. pallipes* populations on the first two axes. Populations of Saint Romain (GAR-2), Louet (ADO-11), Lys (ADO-12), Sélignac (RHO-36) and Mezayon (RHO-39), with an intermediate genetic composition, are circled.

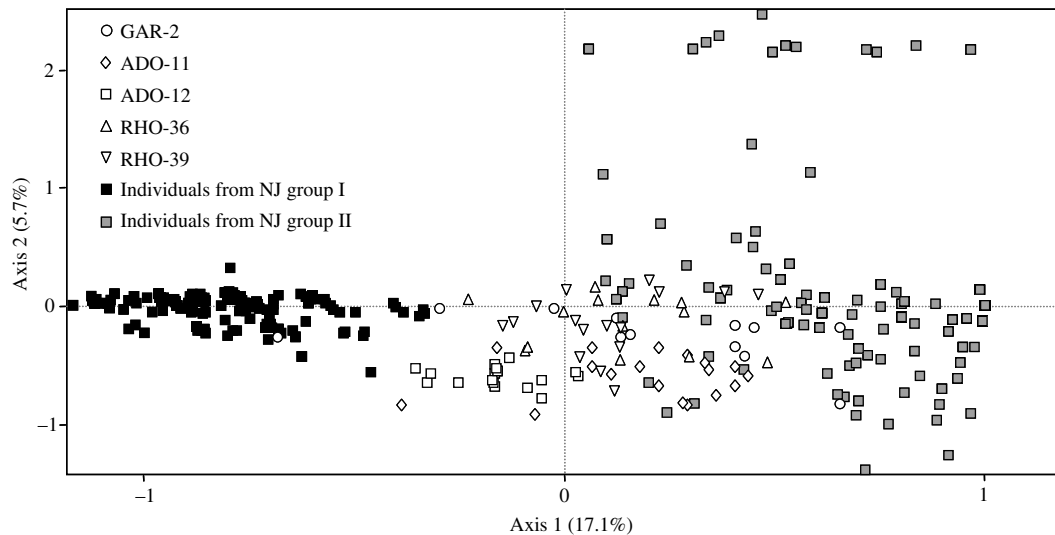


Fig. 5 Correspondence analysis: projection of the individual genotypes on the first two axes. GAR-2 = Saint Romain, ADO-11 = Louet, ADO-12 = Lys, RHO-36 = Sélignac, RHO-39 = Mezayon.

Possible refugial areas for *A. pallipes*

Based on the analysis of mtDNA by RFLP, Grandjean & Souty-Grosset (2000) showed the existence of two genetically differentiated entities of *A. pallipes* in France corresponding to northern and southern populations. The same dichotomy was revealed in the present study using microsatellite markers (see Fig. 2), with stepwise-like mutations contributing significantly to the genetic differentiation between these two groups. Recent studies have demonstrated that microsatellite markers can be effective for discerning historical relationships of divergent populations (Angers & Bernatchez, 1998; Goodman, 1998; Koskinen *et al.*, 2002; Hardy *et al.*, 2003; Fraser & Bernatchez, 2005). According to Estoup & Angers (1998), complete genetic isolation for 2000 generations would constitute a critical value below which the mutation process for microsatellite markers would have a limited influence on population structuring. As *A. pallipes* has a generation time of approximately 4 years, our results suggest that the separation between northern and southern populations exceeded 8000 years, which would place it near the last glaciation period of the Pleistocene. This assumption is further supported by the 1.25% average haplotype divergence found between northern and southern populations by Grandjean & Souty-Grosset (2000), which is consistent with divergence beginning in the

Pleistocene (Avisé, 1998). Therefore, the congruence between results obtained from these different molecular markers clearly confirms the north/south subdivision of *A. pallipes* French populations. According to the present distribution of *A. pallipes*, the hypothesis of two refuges during the last glacial, one on the Atlantic coast and one on the Mediterranean coast, from which the post-Pleistocene dispersal would have occurred seems very likely and is in agreement with hypotheses already proposed for freshwater species distributed in western Europe (Nagel, 2000; Bernatchez, 2001). In brown trout *Salmo trutta* (Linnaeus 1758), the best-studied freshwater species in Europe, two lineages have also been described in France: a Mediterranean lineage which is distributed in river drainages along the Mediterranean coast and in the main Rhône river drainage and an Atlantic lineage distributed in the Garonne, the Loire, the Seine, the Rhine and the upper Rhône river systems (Garcia-Marin, Utter & Pla, 1999; Bernatchez, 2001; Antunes *et al.*, 2002). As these two species (*S. trutta* and *A. pallipes*) share the same habitat, we can postulate that they probably shared these two refugial areas during the Pleistocene. However, given that Spain harbours only populations of the species *A. italicus* (Grandjean *et al.*, 2000, 2001), this eliminates the possibility of a northern expansion of *A. pallipes* from a refuge in Atlantic drainages of Iberia (Garcia-Marin *et al.*, 1999). According to Aurelle *et al.* (2002), south-

western France was ice-free during the last glacial events and this area could have been a possible glacial refuge for brown trout. It is therefore very likely that the Atlantic refuge for *A. pallipes* was located in the Adour and the Garonne rivers as well. Lastly, our results clearly reject the hypothesis of a third refuge for *A. pallipes* in the Rhine river system suggested by Gouin *et al.* (2001) from analysis of RAPD marker data. As revealed in the present study, north-eastern populations are not genetically differentiated, and the pattern observed by Gouin *et al.* (2001) could have been an artefact because of the limited number of populations sampled in northern France.

Secondary contacts between A. pallipes lineages

There is some evidence from our data that genetic introgression between the Atlantic and the Mediterranean lineages might have occurred in five populations belonging to the Garonne, the Adour and the lower Rhône river basins. The strongest evidence was found in the population of Saint Romain (GAR-2) where the CA unambiguously revealed the presence of individuals from each lineage associated with individuals with intermediate genotypes. None of these populations displayed significant Hardy–Weinberg or linkage disequilibria, which indicates that genetic introgression was probably not very recent (Poteaux, Bonhomme & Berrebi, 1999; Beaumont *et al.*, 2001). Indeed, population disequilibria should be negligible after a few generations of random mating (Barton, 2000). The fact that these populations reached equilibrium also indicates that there was no apparent reproductive isolation and genetic barrier to gene flow between crayfish from these two lineages (Barton & Hewitt, 1985; Barton & Gale, 1993).

Whether past secondary contacts between the Atlantic and the Mediterranean lineages were natural or artificial is difficult to answer. However, compared with the brown trout model, the presence of crayfish from the Mediterranean lineage in the Garonne and the upper Loire river systems, suggesting the colonisation of south-western France from a Mediterranean refuge, is surprising. In fish, and especially in trout, natural secondary contacts did not occur between Mediterranean and Atlantic lineages in southern France (Bernatchez, 2001; Aurelle *et al.*, 2002). Even if *A. pallipes* has the ability to walk, which could have allowed this species to cross obstacles that fishes

could not and thereby colonise south-western France, this species would have still used pathways having at least shallow waters; shallow waters that a fish like the brown trout could have easily employed. Moreover, no major difference in habitat requirements has been revealed between populations throughout its distribution area (Neveu, 2000; Grandjean, Momon & Bramard, 2003; Trouilhé *et al.*, 2003) and the French climate varies gradually from north to south with no drastic change that could explain such a distribution through ecological replacement. In fact, a rational hypothesis is that crayfish from the Mediterranean stock could have been introduced in the upper part of the Garonne and the Loire river systems. Human-mediated translocation of white-clawed crayfish appears indeed to have been a common practice throughout western Europe (Souty-Grosset *et al.*, 1997; Largiadèr *et al.*, 2000; Grandjean *et al.*, 2001; Gouin *et al.*, 2003; Fratini *et al.*, 2005; Trontelj, Machino & Sket, 2005) and particularly in France (Machino *et al.*, 2004). It is therefore plausible that crayfish could have been translocated from east to west, leading to the establishment of new populations or to genetic admixture between Atlantic and Mediterranean stocks. The translocation of specimens from a Mediterranean lineage would not be specific to *A. pallipes* since it has also been reported locally in brown trout as a result of stocking events and in freshwater mussels (Berrebi *et al.*, 2000; Nagel, 2000).

Population genetic structure between and within rivers

Hydrography has a strong impact on the genetic structure of most species colonising continental freshwaters (Ward, Woodwark & Skibinski, 1994; Hébert *et al.*, 2000; Costello *et al.*, 2003). While previous studies failed to detect any influence of hydrography on the distribution of the genetic variation in *A. pallipes* (Grandjean & Souty-Grosset, 2000; Gouin *et al.*, 2001), the microsatellite markers used here revealed that river systems had in fact a significant impact on population differentiation. Although we suspect artificial secondary contacts between crayfishes from the Atlantic and the Mediterranean refuges in south-western France, the high degree of genetic differentiation observed between river basins in southern populations indicates that southern populations were probably highly structured prior to these events. The fact that the differentiation between populations of a

river basin was as high as or higher than differentiation between river basins could be explained by two, not necessarily exclusive, hypotheses: (i) the translocation of crayfish from different river basins, as revealed between the two lineages in the present study and (ii) a common origin of populations of different river basins followed by little gene flow within basins.

The high degree of genetic differentiation within river basins revealed by microsatellite analysis strongly suggests the absence of significant gene flow between populations and confirms the previous observations made from mtDNA and RAPD markers (Grandjean *et al.*, 1997; Grandjean & Souty-Grosset, 2000; Gouin *et al.*, 2001). Habitat fragmentation because of the degradation of water quality has progressively confined *A. pallipes* populations to headwater river systems (Changeux, 2003). These populations are now isolated and thus increasingly subject to the effects of random genetic drift including rapid loss of genetic variability (Hartl & Clark, 1997; Amos & Harwood, 1998). Therefore, the various levels of heterozygosity observed in the French populations could be due to differences in local population sizes. The proportional relationship between intra-population genetic variability and population size has been observed in numerous species (Frankham, 1996) and was proposed by Gouin *et al.* (2001) to explain the variation in intra-population diversity in *A. pallipes* revealed with RAPD markers.

However, two additional factors capable of exacerbating the impact of genetic drift in *A. pallipes* populations through bottleneck events have to be considered: demographic fluctuations and human-mediated translocations of crayfish. Concerning the first factor, variation in population size because of demographic and environmental stochasticity reduces effective population size and accentuates the loss of genetic variability (Sherwin & Moritz, 2000; Frankham, Ballou & Briscoe, 2002). In the white-clawed crayfish, populations can experience drastic reductions in size during severe droughts and periodic chemical pollution events (pesticides, fertilisers). For example, La Rune (Vienne, France) was polluted in 1999 with a chlorinated hydrocarbon pesticide, which killed an estimated 4520 *A. pallipes*, virtually the entire population. The following year ten crayfish that had escaped the pollution were discovered in a small affluent, from which the main brook is being

recolonised. There is no doubt that bottlenecks of this magnitude can have a strong impact on the genetic diversity of *A. pallipes* populations. Uncontrolled translocations of crayfish can also have a significant impact on the genetic differentiation of white-clawed crayfish populations (Lörtscher *et al.*, 1997; Santucci *et al.*, 1997; Grandjean *et al.*, 2001; Gouin *et al.*, 2003; Fratini *et al.*, 2005). In a study of Irish populations, Gouin *et al.* (2003) showed that a succession of founder effects because of step-by-step translocations of crayfish from the south to north of Ireland was probably responsible for the observed clinal reduction of genetic diversity. There is also evidence that translocations of a limited number of animals can lead to a severe decrease in heterozygosity relative to the source population (Broders *et al.*, 1999; Williams *et al.*, 2002) and eventually to the complete loss of genetic variation (Grandjean *et al.*, 2001). Thus, demographic fluctuations and human translocations could explain the absence of genetic polymorphism in some French populations sampled for this study. However, it must be kept in mind that an important role of recurrent bottlenecks does not rule out the effects of selective forces in shaping the genetic differentiation of *A. pallipes* populations. More field studies are now needed to gather ecological, morphological and behavioural data that will allow the detection of local adaptation as a factor in genetic structuring of this species.

Conservation implications

A basic prerequisite for managing biodiversity is the identification of population groups with independent evolutionary histories. In this study, our data from microsatellite markers provide strong evidence of historical population differentiation between northern and southern French populations of *A. pallipes*, which is consistent with the results from mtDNA and RAPDs (Grandjean & Souty-Grosset, 2000; Gouin *et al.*, 2001). Therefore, we propose that these entities should be considered as two distinct ESUs according to Moritz (1994). Despite the fact that this approach ignores adaptive differences (Crandall *et al.*, 2000), the evidence from genetic markers, that these two groups of populations have diverged since the late Pleistocene, is a strong argument to classify them as two separate units for management objectives because they represent two evolutionarily

distinct lineages (Avisé, 1994; Moritz, 1994; Fraser & Bernatchez, 2001).

Making further suggestions based on the sole use of neutral genetic markers would be risky at this point. However, such markers can be helpful in providing precious information about population subdivision and genetic variability that could further be used for management purposes. For example, it is clear from our study that the degradation of freshwater habitats has isolated *A. pallipes* populations, making them more sensitive to stochastic environmental changes (Lande, 1988; Sherwin & Moritz, 2000) and more vulnerable to extinction due to loss of genetic variation (Lynch, Conery & Burger, 1995; Frankham *et al.*, 2002). The microsatellite markers even revealed very low levels of genetic variation in several populations, suggesting that they are particularly at risk and they should be given special attention. However, it becomes critical to develop better approaches to identify populations with high priority for conservation, which could combine data about population size and microsatellite variation as recently implemented in a freshwater mussel (Geist & Kuehn, 2005). We also demonstrated that the genetic variation in *A. pallipes* populations was significantly distributed between rivers within each lineage. This result suggests that populations of each group could be managed by river basin if actions had to be taken quickly. Indeed, river basins represent an obvious management target in freshwater species because they constitute a structure in which gene flow has been confined for thousands of years. Such an approach would limit the risks of mixing genes from different pools, as suggested by Berrebi (1997).

In conclusion, this study provided new insights into the biogeography of *A. pallipes* and valuable data that can be used for its conservation in France. However, the discovery of mixed populations poses some challenges for future management strategies (Allendorf *et al.*, 2001). More research needs to be carried out in order to evaluate the extent of genetic admixture between the Atlantic and the Mediterranean lineages and its consequences for southern populations. The analyses of genetic admixture implemented in this study must be improved because of the low polymorphism of the microsatellites and the lack of diagnostic markers. Therefore, more microsatellite markers would be needed, which would in addition allow the use of statistical tools that are more

powerful analytically (Pritchard, Stephens & Donnelly, 2000; Choisy, Franck & Cornuet, 2004). However, this task will remain challenging as there is no record of the translocation events and potential parental populations are not known. It will also become critical to collect data concerning local adaptation in isolated populations in order to avoid any risk associated with mixing complexes of genes adapted to different environments and also to preserve the components of adaptive significance in this species (Crandall *et al.*, 2000).

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Appendix 1 Allele frequencies for each microsatellite locus in each population

Population		Locus																							
		GAR-1	GAR-2	GAR-3	GAR-4	GAR-5	GAR-6	GAR-7	GAR-8	GAR-9	GAR-10	ADO-11	ADO-12	AUD-13	AUD-14	HER-15	HER-16	ORN-17	CHA-18	SEV-19	SEV-20	SEV-21	LOI-22		
<i>Ap2</i>																									
184	-	0.125	-	-	-	0.500	-	1.000	-	-	0.053	0.306	-	-	-	-	-	-	0.045	0.053	0.417	0.175	0.517	-	
186	-	0.542	1.000	1.000	1.000	-	1.000	1.000	1.000	0.643	0.900	0.526	0.028	1.000	1.000	1.000	1.000	-	-	-	-	-	-	-	-
190	1.000	0.333	-	-	-	0.500	-	-	-	0.143	0.100	0.421	0.667	-	-	-	-	1.000	0.955	0.947	0.583	0.825	0.483	-	
192	-	-	-	-	-	-	-	-	-	0.214	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>Ap3</i>																									
148	-	0.458	0.954	0.775	1.000	-	1.000	0.974	0.714	0.900	0.210	0.139	1.000	1.000	0.929	1.000	-	-	-	-	-	-	-	-	
150	1.000	0.542	0.045	0.225	-	1.000	-	0.026	0.250	0.100	0.789	0.864	-	-	0.071	-	1.000	1.000	0.921	0.979	0.925	1.000	1.000	-	
152	-	-	-	-	-	-	-	-	0.036	-	-	-	-	-	-	-	-	-	-	-	0.021	-	-	-	
154	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.079	-	0.075	-	-	
<i>Ap5</i>																									
331	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.026	-	-	
335	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.658	0.292	0.553	0.017	-	
337	0.775	0.083	-	-	-	0.454	-	-	-	-	-	-	-	-	-	-	-	1.000	0.727	0.342	0.708	0.263	0.655	-	
339	0.200	0.125	0.100	-	-	0.045	-	-	-	-	-	0.156	-	-	-	-	-	-	0.273	-	-	-	0.241	-	
341	0.025	0.500	0.900	0.875	0.722	0.500	0.975	1.000	0.808	0.800	0.737	0.719	1.000	0.944	1.000	0.941	-	-	-	-	-	0.158	0.086	-	
343	-	0.292	-	0.125	0.278	-	0.025	-	0.192	0.200	0.263	0.125	-	0.056	-	0.059	-	-	-	-	-	-	-	-	-
345	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
347	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>Ap6</i>																									
344	0.700	0.833	1.000	1.000	1.000	0.364	1.000	1.000	0.821	0.900	0.790	0.972	1.000	1.000	1.000	1.000	1.000	1.000	0.546	0.684	0.708	0.850	0.633	-	
348	0.300	0.167	-	-	-	0.636	-	-	0.179	0.100	0.210	0.028	-	-	-	-	-	-	0.454	0.316	0.292	0.150	0.367	-	
<i>Ap7</i>																									
256	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.033	
258	-	-	-	-	-	-	-	-	0.038	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
260	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
262	-	-	-	-	-	0.050	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
264	0.868	0.297	-	-	-	0.950	-	-	-	-	-	-	-	-	-	-	-	-	1.000	0.868	1.000	0.722	0.833	-	
266	0.105	0.250	-	-	-	0.050	-	-	0.269	0.300	0.447	0.861	-	-	-	-	-	0.454	1.000	0.132	-	0.250	0.133	-	
268	-	-	-	-	-	-	-	-	-	-	0.026	-	-	-	-	-	-	-	-	-	-	0.028	-	-	
272	-	-	-	-	-	-	-	-	0.038	0.400	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
274	0.026	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
276	-	0.083	-	-	-	0.500	-	-	0.385	0.100	0.394	0.139	-	-	-	-	-	-	-	-	-	-	-	-	
278	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
280	-	0.375	1.000	1.000	0.450	-	1.000	0.806	0.269	0.200	0.132	-	1.000	1.000	0.929	1.000	-	-	-	-	-	-	-	-	
282	-	-	-	-	-	-	-	0.194	-	-	-	-	-	-	0.071	-	-	-	-	-	-	-	-	-	
284	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
290	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Appendix 1 (Continued)

Population		LOI-23	LOI-24	LOI-25	LOI-26	LOI-27	LOI-28	LOI-29	LOI-30	RHI-31	RHI-32	RHO-33	RHO-34	RHO-35	RHO-36	RHO-37	RHO-38	RHO-39	RHO-40	RHO-41	RHO-42	SEI-43	SEI-44
Ap2		184	0.318	0.237	-	-	-	0.222	0.300	0.344	0.208	0.187	-	-	-	-	-	-	-	-	-	0.710	0.500
		186	-	-	1.000	1.000	1.000	-	-	-	0.333	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-
		190	0.682	0.763	-	-	-	0.778	0.700	0.656	0.458	0.812	-	-	-	-	-	-	-	-	-	0.290	0.500
		192	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ap3		148	0.250	-	1.000	0.750	0.300	0.964	0.893	0.425	-	0.187	-	0.094	0.150	0.067	0.028	0.150	0.250	0.187	-	-	0.300
		150	0.545	1.000	-	0.250	0.700	0.036	0.107	0.575	1.000	0.687	1.000	0.906	0.850	0.933	0.917	0.850	0.750	0.812	0.947	0.700	-
		152	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.055	-	-	-	-	-	-
		154	0.205	-	-	-	-	-	-	-	0.125	-	-	-	-	-	-	-	-	-	-	0.053	-
Ap5		335	0.595	-	-	-	-	-	-	-	-	0.083	0.156	-	0.045	-	-	-	-	-	-	-	-
		337	0.404	0.750	-	-	-	0.687	0.600	0.437	0.458	0.562	0.031	-	-	0.375	0.237	-	-	-	0.789	0.350	
		339	-	0.219	-	-	-	0.312	0.400	0.406	0.542	0.354	0.219	0.300	-	0.625	-	-	-	-	0.211	0.650	
		341	-	0.031	-	0.850	0.800	0.821	0.679	0.825	-	0.156	-	0.594	0.700	0.864	-	0.763	0.125	0.500	-	-	
		343	-	-	0.150	0.200	0.179	0.321	0.175	-	-	-	-	-	-	0.091	-	-	-	0.500	-	-	
		345	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.725	-	-	
		347	-	-	0.667	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.150	-	-	
Ap6		344	1.000	0.656	0.857	1.000	1.000	1.000	1.000	0.550	0.344	0.125	0.396	0.875	1.000	1.000	1.000	1.000	1.000	1.000	0.921	1.000	
		348	-	0.344	0.143	-	-	-	0.889	0.450	0.656	0.604	0.125	-	-	-	-	-	-	-	-	0.079	
Ap7		256	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
		258	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
		260	-	-	-	-	-	-	-	-	0.042	-	-	-	-	-	0.056	-	-	-	-	-	
		262	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.111	-	-	-	-	-	
		264	0.932	1.000	-	-	0.214	0.154	-	1.000	0.900	1.000	0.958	1.000	0.219	0.233	0.139	0.275	0.475	-	0.944	1.000	
		266	0.068	-	-	-	-	0.038	-	-	-	-	-	0.437	-	-	-	-	-	-	0.056	-	
		268	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
		272	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
		274	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.400	-	0.075	-	-	-	
		276	-	-	-	-	-	0.250	0.423	-	-	-	-	-	-	-	0.250	-	-	-	-	-	
		278	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
		280	-	-	1.000	0.944	0.950	0.536	0.385	0.974	-	-	-	0.125	-	0.333	0.333	0.725	0.450	1.000	-	-	
		282	-	-	-	0.056	0.050	-	-	0.026	-	-	-	0.031	0.900	-	0.111	-	-	-	-	-	
		284	-	-	-	-	-	-	-	-	-	-	-	-	0.050	0.033	-	-	-	-	-	-	
		290	-	-	-	-	-	-	-	-	-	-	-	0.187	-	-	-	-	-	-	-	-	