MITOCHONDRIAL AND MICROSATELLITE DNA ANALYSES OF *AUSTROPOTAMOBIUS PALLIPES* POPULATIONS IN SOUTH TYROL (ITALY) AND TYROL (AUSTRIA)

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ABSTRACT

Historical data show that the native white-clawed crayfish, *Austropotamobius pallipes*, was once widespread throughout South Tyrol (northern Italy), whereas recent studies identified only half a dozen remaining populations. In order to implement conservation strategies based on knowledge of genetic diversity, each of the six remaining populations in South Tyrol and one population in Tyrol (Austria) were investigated by (i) sequencing segments of two mitochondrial DNA genes, 16S rRNA and cytochrome c oxidase subunit I, and (ii) by analysing four microsatellite DNA loci. Extremely low degrees of genetic differentiation within and among the South Tyrolean populations of *A. pallipes* were found with mitochondrial DNA sequences. In contrast, microsatellite data displayed not only substantial genetic structure among populations, but also moderate genetic variability within four out of six populations in South Tyrol. The two remaining populations revealed a complete absence of genetic variability. Moreover, both of these populations as well as the population from Austria were fixed for single alleles at three of the four investigated microsatellite loci. Our data have important conservation implications and also show that mitochondrial DNA is not always a sufficient tool to study crayfish populations on a small geographical scale.

Key-words: white-clawed crayfish, genetic diversity, microsatellite DNA, mitochondrial DNA.

ANALYSES DE L'ADN MITOCHONDRIAL ET DES MICROSATELLITES DES POPULATIONS D'*AUSTROPOTAMOBIUS PALLIPES* DANS DU TYROL DU SUD (ITALIE) ET DANS LE TYROL (AUTRICHE)

RÉSUMÉ

Des données historiques montrent que l'écrevisse à pattes blanches, *Austropotamobius pallipes*, était fortement représentée dans tout le Tyrol du Sud (nord de l'Italie). Cependant, de récentes études portent à une demi-douzaine les populations

encore existantes. Dans le cadre de la mise en place d'une stratégie de conservation, la diversité génétique de chacune de ces six populations ainsi que d'une population du Tyrol autrichien a été analysée (i) par séquençage de portions de deux gènes mitochondriaux, codant pour l'ARN 16S et la cytochrome oxydase I et (ii) par analyse de quatre loci microsatellites. Une très faible différenciation génétique a été trouvée dans et entre les populations du Tyrol du Sud avec le marqueur mitochondrial. Au contraire, les données microsatellites montrent non seulement une substantielle structure génétique entre les populations, mais aussi une variabilité modérée dans quatre des six populations du Tyrol du Sud. De plus, deux de ces populations ainsi que celle d'Autriche montrent une fixation d'allèles pour trois des quatre loci microsatellite analysés. Nos données apportent d'importantes implications pour la conservation de la diversité génétique et montrent, également que l'ADN mitochondrial n'est pas toujours adapté pour étudier les populations d'écrevisses sur une petite échelle géographique.

Mots-clés : écrevisse à pattes blanches, diversité génétique, ADN microsatellite, ADN mitochondrial.

INTRODUCTION

Freshwater invertebrates and fishes are vulnerable to habitat loss and pollution, because of destruction and contamination of freshwater ecosystems (DYNESIUS and NILSSON, 1994). The loss of habitats and the subsequent fragmentation of populations is one of the most important factors in species extinction, as fragmentation usually reduces rates of gene flow among subpopulations and leads to a decrease in effective population size (NEY-NIFFLE and MANGEL, 2000). Small, isolated populations are particularly prone to loss of genetic variability through the action of genetic drift. This can have negative consequences on the long-term viability of a population, since genetic variability represents an important potential to cope with and adapt to changing environments.

In Europe, autochthonous freshwater crayfish belong to the most endangered aquatic species. Populations of the white-clawed crayfish, *Austropotamobius pallipes*, have declined by 25-50% over the last 25 years (BERNARDO, ILHEU and COSTA, 1997; GHERARDI, SOUTY-GROSSET and REYNOLDS *et al.*, 2003; HOLDICH and ROGERS, 1997; SOUTY-GROSSET, GRANDJEAN and GOUIN, 2003) and a similar trend for this species can also be observed in the northern Italian province of South Tyrol (Autonomous Province of Bozen/Bolzano). According to historical records the autochthonous species *A. pallipes* was once widely distributed in several areas of the region. About 50 different localities with crayfish occurrences are known from the literature, which were mainly located in the broader main valleys and on higher plateaus (FÜREDER and MACHINO, 1999). Today, white-clawed crayfish have been extirpated from the majority of these water bodies and the ongoing trend of dramatic population decline has been well documented in a series of recent studies (FÜREDER and MACHINO, 1998, 1999; FÜREDER, OBERKOFLER and MACHINO, 2002, FÜREDER *et al.,* 2003). The main causes of the loss of crayfish populations in South Tyrol are destruction and alteration of habitats, water pollution, and the stocking with alien crayfish and predatory fish species (FÜREDER *et al.*, 2002).

Quite recently, an action plan was elaborated in order to preserve the white-clawed crayfish from complete extinction in South Tyrol (FÜREDER *et al.*, 2002, 2003). The most important measures include the enhancement of environmental conditions of existing crayfish populations by wastewater treatment and the reduction of pesticides and fertilisers, and the protection and improvement of habitat structure. In addition, renaturation of suitable historical habitats as well as breeding and reintroduction programs were suggested (FÜREDER *et al.*, 2002) and are currently implemented.

In order to define efficient conservation strategies, accurate data on systematics and population structure are crucial (SOUTY-GROSSET *et al.*, 1997, SOUTY-GROSSET,

GRANDJEAN and GOUIN, 2003). Although *A. pallipes* populations were shown to display great degrees of morphological and genetic diversity in the Alpine region (LARGIADÈR *et al.*, 2000; LÖRTSCHER *et al.*, 1997; SANTUCCI *et al.*, 1997), nothing is known about the phylogenetic status and genetic composition of crayfish populations in South Tyrol. Therefore we addressed these important questions and investigated six *A. pallipes* populations from South Tyrol (Italy) and one population from Tyrol (Austria) by analysing variation in mitochondrial DNA gene sequences and highly variable microsatellite markers.

MATERIALS AND METHODS

Molecular techniques

Specimens of *A. pallipes* were sampled from six sites in South Tyrol (northern Italy) and one site in Tyrol (western Austria) during summer in 2002 and 2003 (Figure 1 and Table 1). Collecting was performed either by hand during the night or with crayfish traps. A non-destructive sampling procedure was employed by taking the third pereiopod from each individual. The legs were preserved separately in absolute ethanol and the animals released at the sampling locality.

Total nucleic acid was isolated from muscle tissue according to the procedure of SAMBROOK, FRITSCH and MANIATIS, (1989). A preliminary study was carried out to evaluate the suitability of mitochondrial DNA (mtDNA) sequences for the investigation of *A. pallipes* populations on a small geographical scale (Table 1). A segment of the mtDNA 16S rRNA gene (16S) was amplified with primers 1472 and 1471 (CRANDALL and FITZPATRICK, 1996). Primer combination ORCOIF (TAYLOR and HARDMAN, 2002) and H7188 (WILLIAMS *et al.*, 2001) was used in a first step to amplify and sequence a segment of the mtDNA cytochrome c oxidase subunit I (COI). The sequences from nine *A. pallipes* specimens from three populations were then aligned to design the new primer Ap-COI-

Table I

**Sampling sites and number of individuals analysed for each population of *A. pallipes*.**

Tableau I

Sites d'échantillonnage et nombre d'individus analysés dans chaque population d'*A. pallipes*.

No	Sampling site	Site code	No of analysed specimens		
			Sequences		Microsatellites
			16S	CO I	4 loci
1	Angelbach [a]	ANG	7	8	39
2	Ritscherbach [a]	RIT	10	10	40
3	Krebusbach [a]	KRB	10	10	31
4	Hyppolithbach [a]	HYP	6	10	39
5	Krebsbach Lana [a]	KBL	1	1	6
6	Gießbach [a]	GIB	1	1	9
7	Plansee [b]	PLS	3	3	35
		Total	38	43	199

[a] South Tyrol (Italy).
[b] Tyrol (Austria).
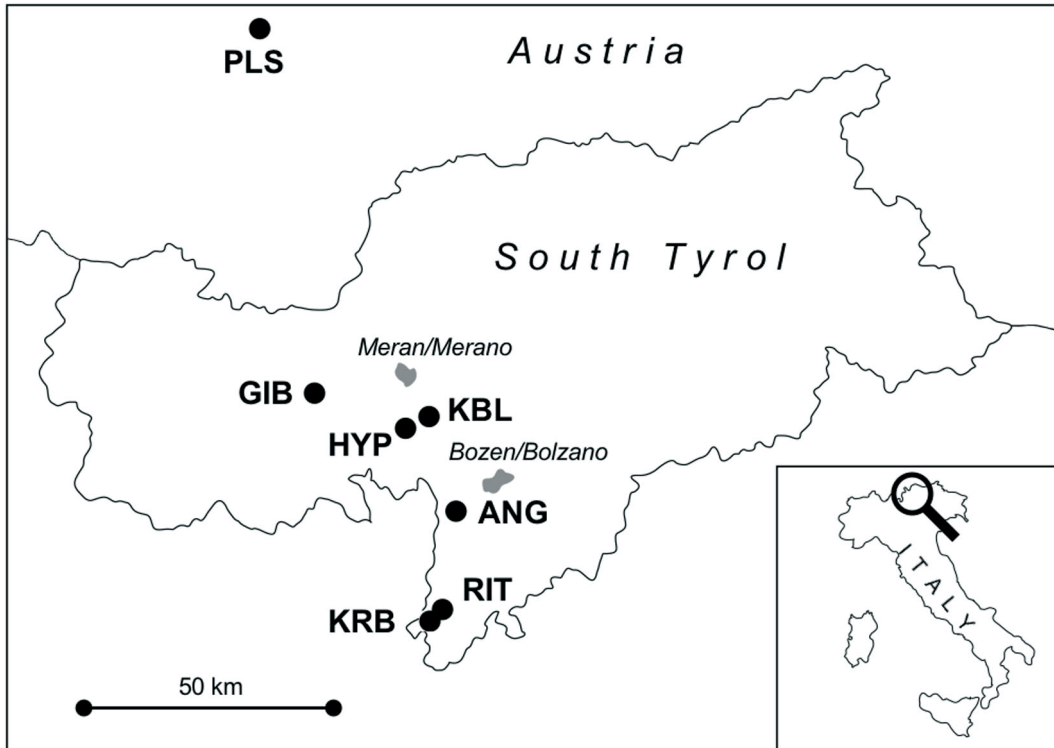
Figure 1
   Sampling sites in South Tyrol (Italy) and Tyrol (Austria).

Figure 1
   Sites d'échantillonnage dans le Tyrol du Sud (Italie) et dans le Tyrol (Autriche).

LB-R (Table 2). For all subsequent amplification reactions of COI primer pair ORCOIF and Ap-COI-LB-R was used.

Ten microliter reaction volumes contained 1 µM of each primer, 200 µM dNTP-Mix (GeneCraft, Germany), 1.5 mM $MgCl_2$, 0.5 U BioTherm DNA polymerase (GeneCraft) and 2 µl DNA template. Amplification reactions were run on the Mastercycler Gradient (Eppendorf, Germany) or GeneAmp PCR System 2700 (Applied Biosystems, USA) under the following conditions: 2 min initial denaturation at 94°C followed by 40 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 1 min, and a final extension step at 72°C for 5 min. After separation and visualization on a 1.5% ethidium bromide stained agarose gel amplification products were purified using Montage PCR Centrifugal Filter Devices (Millipore, USA) and quantified with a spectrophotometer (BioPhotometer, Eppendorf) at 260/280 nm.

Cycle sequencing was carried out in 10 µl reaction volumes with the CEQ DTCS Quick Start Kit (Beckman Coulter, USA) and the recommended amount of purified PCR product. In addition to the two amplification primers, an internal sequencing primer COIF was used (Table 2). The cycling conditions were set to 30 cycles of 20 s at 96°C, 20 s at 50°C and 4 min at 60°C. Sequencing products were separated and detected on the CEQ 8000 Genetic Analysis System (Beckman Coulter).

Four microsatellite loci, Ap1, Ap2, Ap3 and Ap6 (GOUIN, GRANDJEAN and SOUTY-GROSSET, 2000), were employed to additionally assess the genetic structure of *A. pallipes* populations. Each forward primer was fluorescently labelled with a Beckman WellRED dye (Beckman Coulter; Table 2). Ten microliter amplification reaction volumes contained 200 nM

Table II
Primers used to analyse mtDNA and microsatellite loci in *A. pallipes* populations.

Tableau II
Amorces utilisées pour l'analyse de l'ADN mitochondrial et des loci microsatellites dans les populations d'*A. pallipes*.

	Primer name	Primer sequence (5'-3')	Reference
mt DNA			
16S	1471	CCTGTTTA(AGCT)CAAAAACAT	CRANDALL and FITZPATRICK, 1996
	1472	AGATAGAAACCAACCTGG	CRANDALL and FITZPATRICK, 1996
COI	COIF	CCAGCTGGAGGAGGAGA(CT)CC	WILLIAMS *et al.*, 2001
	H7188	CATTTAGGCCTAAGAAGTGTTG	WILLIAMS *et al.*, 2001
	ORCOIF	AACGCAACGATGATTTTTTTCTAC	TAYLOR and HARDMAN, 2002
	Ap-COI-LB-R	AAACAAAGGAAATCCATGAAC	designed for this study
Microsatellite locus			
Ap1	Ap1-F	D2 - TCTTGGGGATTGGCTAGTTG	GOUIN *et al.*, 2000
	Ap1-R	CCTGAACTAAAAGGTGCTTTGG	÷
Ap2	Ap2-F	D3 - TTCGATATAACCGTTTGACCTG	÷
	Ap2-R	TCAGACTTTGGCCATTGAAG	÷
Ap3	Ap3-F	D4 - CGCCTATCTAACCTTGGTTGTC	÷
	Ap3-R	GGACTTGGGAAGCCTTGTG	÷
Ap6	Ap6-F	D3 - GCTGTGTGGGATGGAGGT	÷
	Ap6-R	CACTAGCGTATTCAAGCAACT	÷

D2, D3, D4... Beckman WellRED fluorescent dyes.

(Ap1/Ap3 multiplex reaction) or 400 nM (Ap2 and Ap6) of each primer, 200 µM dNTP-Mix (GeneCraft), 1.5 mM MgCl$_2$, 0.5 U HotMaster Taq DNA polymerase (Eppendorf) and 2 µl of DNA template. The thermocycler was programmed to perform an initial denaturation step of 2 min at 94°C followed by 30 to 35 cycles of 30 s at 94°C, 30 s at 60°C (Ap1/Ap3 and Ap6) or 54°C (Ap2) and 45 s at 65°C, and a final extension of 10 min at 65°C.

Each sample was run with an internal size standard on the CEQ 8000 Genetic Analysis System (Beckman Coulter). Sizing of fragments and assignment to particular size classes was performed by using the Fragment Analysis Software of the same manufacturer. To control the validity of the sizing and verify the assignment to size classes, all alleles allocated to a particular class were re-examined by eye.

Data analyses

Partial sequences of each of the two mtDNA genes were aligned by eye using the computer program SEQUENCER Version 4.2 for Windows (Gene Codes Corporation,

Ann Arbor, USA) and unique haplotypes were identified. The 16S-dataset was expanded with additional sequences of *A. pallipes* from GenBank (accession numbers AF237590-AF237610 and AJ242700-AJ242711), which covered the whole distribution range of the species. PAUP* version 4.0 Beta (SWOFFORD, 2002) was used to calculate pairwise distances among sequences. Gaps were treated as missing data.

Genetic variability of the four microsatellite loci was estimated for each population in terms of the number of alleles (N_A), the number of genotypes (N_G), allele frequencies, and the observed (H_O) and expected heterozygosity (H_E). Departure from Hardy-Weinberg equilibrium was tested according to the procedure of GUO and THOMPSON (1992) using a Markov chain Monte Carlo series of permutations, as implemented in ARLEQUIN version 2.0 (SCHNEIDER *et al.*, 2000). Genotypic linkage disequilibrium was tested for all pairs of polymorphic loci in each population as well as across populations with the software GENEPOP version 3.3 (RAYMOND and ROUSSET, 1995), which performs Fisher's exact test by using a Markov chain (GUO and THOMPSON, 1992).

Exact probability tests for genotypic and genic differentiation among all pairs of populations were conducted by using GENEPOP version 3.3 (RAYMOND and ROUSSET, 1995). The computer software ARLEQUIN version 2.0 (SCHNEIDER *et al.*, 2000) was used to calculate pairwise F_{ST} values among all population pairs, as an additional measure of population subdivision (SLATKIN, 1995). All significance values resulting from multiple comparisons were corrected for type I errors by applying the sequential Bonferroni procedure (RICE, 1989).

RESULTS

The alignment of the 16S sequences comprised 430 nucleotide positions. No base pair substitutions were observed among the sequences of all 38 specimens of *A. pallipes* (Table 1). The only variability was detected in a poly-adenine region where all individuals from the RIT population had a single nucleotide insert (A) and two individuals from the ANG population had a 3-bp insert (AAA). The most common 16S haplotype (lacking inserts) was identical to haplotype S3, which was deposited in GenBank under the accession number AJ242706 and was found to be distributed in diverse populations of *A. pallipes* in Austria, Switzerland and north-western Italy (LARGIADÈR *et al.*, 2000). Sequences of the COI gene segment were obtained from 43 individuals and the length of the alignment was 1,063 nucleotides. In general, one single haplotype was found to be distributed within and among all populations surveyed. An exception was population KRB, where a second haplotype was found in two individuals, which differed by a single point mutation.

Only population KRB was polymorphic at all the four investigated microsatellite loci, while populations ANG, RIT and HYP were polymorphic at three loci, Ap2, Ap3 and Ap6 (Table 3). Populations KBL, GIB and PLS were fixed for a single allele at loci Ap1, Ap3 and Ap6, while for locus Ap2 between 2 and 4 alleles were found. The overall number of alleles across all loci ranged from 2 to 10. The highest number of private alleles was found in population KRB. Allele frequencies for each locus and population are shown in Figure 2. The average observed heterozygosity across all loci and populations amounted to 0.525 (± 0.1995). Three populations displayed significant deviations from expected
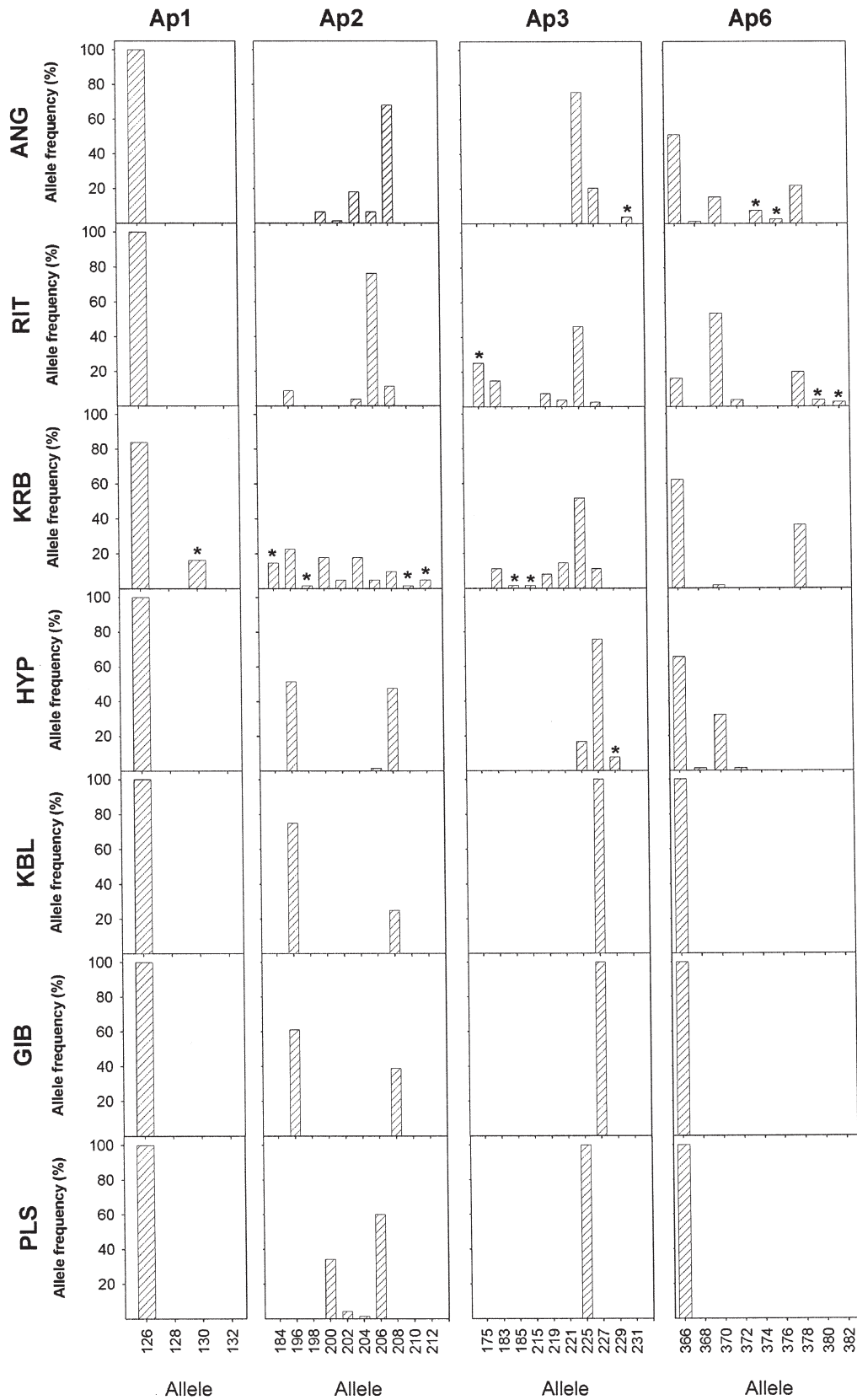
Figure 2 ▶

Allele frequency distributions across microsatellite loci and populations. Asterisks denote private alleles.

Figure 2

Distributions des fréquences d'allèles sur les loci microsatellites et dans les populations. Les astérisques signalent les allèles privées.

MICROSATELLITE LOCUS

Hardy-Weinberg proportions after a sequential Bonferroni correction: KRB at loci Ap3 and Ap6, and HYP at locus Ap6 at the 5% significance level, and ANG at locus Ap6 at the 1% significance level (Table 3). Only population RIT was found to be in Hardy-Weinberg equilibrium at all four loci. Genotypic linkage disequilibrium was tested to verify that there was no association among genotypes at different loci. Tests could be performed for populations ANG, RIT and HYP for the polymorphic loci Ap2, Ap3 and Ap6, and for population KRB for all pairs of loci. The estimated P-values ranged from 0.094 to

Table III
   Summary table of variation detected at four microsatellite loci in seven *A. pallipes* populations. The numbers of alleles (N_A), number of genotypes (N_G), observed (H_O) and expected (H_E) heterozygosity are given. Number of specimens analysed for each population is indicated in parentheses. Asterisks indicate significant deviation from Hardy-Weinberg equilibrium at the 5% level (*) and 1% level (**) after table-wide Bonferroni correction.

Table III
   Tableau résumé des variations détectées sur quatre loci microsatellites dans sept populations d'*A. pallipes*. Les nombres d'allèles (N_A), le nombre de génotypes (N_G), l'hétérozygotie observée (H_O) et attendue (H_E) sont indiqués. Le nombre de spécimens analysés dans chaque population est indiqué entre parenthèses. Les astérisques indiquent une déviation significative de l'équilibre de Hardy-Weinberg de 5 % (*) et de 1 % (**), après correction selon la table de Bonferroni.

Locus		ANG (39)	RIT (40)	KRB (31)	HYP (39)	KBL (6)	GIB (9)	PLS (35)	Mean N_A	Total N_A
Ap1	N_A	1	1	2	1	1	1	1	1.14	2
	N_G	1	1	3	1	1	1	1		
	H_O	-	-	0.258	-	-	-	-		
	H_E	-	-	0.302	-	-	-	-		
Ap2	N_A	5	4	10	3	2	2	4	4.29	10
	N_G	7	5	19	4	2	2	5		
	H_O	0.564	0.400	0.710	0.487	0.500	0.111	0.514		
	H_E	0.537	0.4392	0.8630	0.519	0.409	0.765	0.677		
Ap3	N_A	3	6	7	3	1	1	1	3.14	10
	N_G	4	10	11	5	1	1	1		
	H_O	0.308	0.750	0.548*	0.359	-	-	-		
	H_E	0.389	0.731	0.710	0.535	-	-	-		
Ap6	N_A	6	6	3	4	1	1	1	3.14	9
	N_G	7	9	3	4	1	1	1		
	H_O	0.819**	0.650	0.759*	0.667*	-	-	-		
	H_E	0.667	0.675	0.492	0.476	-	-	-		
Mean N_A/pop		3.75	4.25	5.50	2.75	1.25	1.25	1.75		
Mean H_O		0.56	0.60	0.57	0.50					
Mean H_E		0.53	0.62	0.59	0.51					

⁻ Test could not be performed because locus is monomorphic.

0.857, and thus the null hypothesis of independence among genotypes across all four microsatellite loci was accepted.

The exact tests across all loci and populations revealed significant differences in both allele and genotype frequency between 19 population pairs. For population pair KBL and HYP significant genotypic differentiation was found, while allelic differentiation was not significant. Population pair GIB and KBL showed neither significant genotypic nor allelic differentiation (Table 4). Pairwise F_{ST} values were significantly different from zero in 20 out of 21 pairwise comparisons. Congruent with the results of the exact tests for genotypic and genic differentiation, no significant difference was found between populations GIB and KBL (Table 4). Pairwise F_{ST} estimates ranged from zero to 0.749 with a mean value of 0.342 (± 0.1977).

Table IV

Population pairwise F_{ST} estimates obtained from microsatellite data (below diagonal) and levels of significance for the exact test for population differentiation (genotypic/genic differentiation; above diagonal) after sequential Bonferroni correction.

Tableau IV

Estimations des F_{ST} par paires de populations obtenues à partir des données des microsatellites (en dessous de la diagonale) et niveau de significativité pour le test exact de différentiation des populations (différentiation génotypisue/génique; au-dessus de la diagonale) après correction selon la séquence de Bonferroni.

	ANG	RIT	KRB	HYP	KBL	GIB	PLS
ANG		***/***	***/**	***/***	***/***	***/***	***/***
RIT	0.2733***		***/***	***/***	***/***	***/***	***/***
KRB	0.1279***	0.2263***		***/***	***/***	***/***	***/***
HYP	0.2655***	0.3745***	0.2378***		*/NS	**/*	***/***
KBL	0.4330***	0.5000***	0.2916***	0.1058*		NS/NS	***/***
GIB	0.4126***	0.5045***	0.2974***	0.0917*	– 0.0289		***/***
PLS	0.3596***	0.3960***	0.2773***	0.5515***	0.7491***	0.7394***	

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS... not significant.

DISCUSSION

Analyses of mtDNA sequences and microsatellite DNA variation in *A. pallipes* from South Tyrol did not yield the same results. While almost complete monomorphism was found for 16S and COI sequences, microsatellite patterns revealed genetic diversity within and across the four populations ANG, RIT, KRB and HYP. Such a finding is not surprising considering the differences in the modes of inheritance and mutation rates of mitochondrial and nuclear DNA. So far, several authors studied population structure and phylogeographic distribution patterns of the white-clawed crayfish on a larger geographical scale by employing RFLP analyses of the mtDNA or by sequencing the 16S rRNA gene (GRANDJEAN *et al.*, 2000, 2001, GRANDJEAN, BOUCHON and SOUTY-GROSSET, 2002; GRANDJEAN and SOUTY-GROSSET, 2000; LARGIADÈR *et al.*, 2000). Pronounced population structure was found among different localities in Italy, France and Switzerland, whereas no genetic variation was observed across Spanish populations and among the most northern French and English populations. However, intrapopulation variability was

found to be very low or completely absent. An exception was found for populations in the southern part of France, where mitochondrial and microsatellite markers revealed high levels of within-population variability (SOUTY-GROSSET, GRANDJEAN and GOUIN, 2003).

In general, the distribution patterns of genetic diversity in *A. pallipes* and many other species can be explained by Pleistocene palaeogeographic events (BERNATCHEZ and WILSON, 1998; GRANDJEAN and SOUTY-GROSSET, 2000; HEWITT, 1996; LARGIADÈR *et al.*, 2000). During the ice ages many species were confined to southern refugial zones located on the Balkan, Apennine and Iberian peninsulas (TABERLET *et al.*, 1998). It is believed that after glaciation the white-clawed crayfish recolonised new territories from multiple refugial areas (reviewed in HOLDICH, 2002). The incidence of the same mtDNA 16S haplotype in South Tyrol, and at different localities in Switzerland and north-western Italy could be a consequence of a rapid postglacial recolonisation event from the same refugial population about 15,000-10,000 years ago. The alternative hypothesis of human translocation, which appeared to be a rather common activity in Europe (see MACHINO *et al*., 2004), seems less probable for the South Tyrolean populations, for which microsatellite DNA data displayed certain degrees of genetic variability. Moreover, microsatellite markers revealed significant population differentiation on a small geographical scale. This pattern is either caused by an effective separation of populations and absence of gene flow soon after postglacial colonization of the different brooks, or alternatively by a recent isolation of populations due to the fragmentation of habitats. In the latter case, population differentiation would arise from the stochastic process of genetic drift enhanced by decreases in effective population size.

South Tyrol experienced major landscape changes during the last two centuries (FEUERSTEIN, 1999). River regulation, damming, and the draining of swamps and wetlands were important measures of land reclamation, which provided the fundament for the development of agriculture, but also led to great losses and fragmentation of aquatic habitats (FÜREDER *et al.*, 2003). As a consequence thereof, the few existing crayfish populations in South Tyrol are limited to small isolated stretches of little creeks (FÜREDER, OBERKOFLER and MACHINO, 2002; FÜREDER and MACHINO, 1999). Although the present-day isolation of crayfish populations is an obvious fact, which could be responsible for the formation of the observed patterns of genetic structure, we cannot exclude the possibility that these populations represented genetically differentiated stocks before the initiation of human activities. GOUIN *et al.* (2002) found extensive gene flow for *A. pallipes* within a single brook, showing that this crayfish species is able to spread over remarkable distances of favourable habitat in the absence of a barrier. In contrast, surveys of different streams within the same basin, which were characterized by a limited degree of habitat fragmentation, displayed high degrees of population structure as well as isolation by distance (SOUTY-GROSSET, GRANDJEAN and GOUIN, 2003). Such findings clearly demonstrate the low dispersal ability of the species over longer distances. The assumption of long-term historical population separation in South Tyrol could also be supported by the finding of haplotype variants, which differed only by deletions or insertions in a poly-adenine stretch of the 16S sequence. Given the fact that mutation rates of mtDNA are higher than those of nuclear DNA, and that mononucleotide repeats have an at least ten-fold increased susceptibility to insertion and deletion mutations compared to non-repetitive sequences (DIERINGER and SCHLÖTTERER, 2003), it is probable that these mutations emerged after colonisation of the present habitats. Mitochondrial DNA has a haploid genome and is usually only maternally inherited. The effective population size for mtDNA is approximately 1/4 of that for nuclear DNA genes so that genetic drift is more pronounced and fixation rates are faster. Regarding the RIT population, where all the surveyed individuals displayed a 16S haplotype variant with a single adenosine insert, one could speculate that this haplotype arose by mutation quite recently and was fixed by lineage sorting in the absence of gene flow. Another recent slippage-like mutation event

and a transition base pair substitution could be assumed for the other haplotype variants, with the difference that the original haplotype was not lost via drift, but coexists with the new haplotype.

The genetic diversity in the populations ANG, RIT, KRB and HYP was higher than that found in the largest *A. pallipes* population in the Poitou-Charentes region in France, which was determined with the same microsatellite markers (GOUIN, GRANDJEAN and SOUTY-GROSSET, 2000, GOUIN *et al.*, 2002). While for the French population a mean heterozygosity value of 0.38 was found, the average observed heterozygosity across all loci and populations in South Tyrol amounted to 0.53. The ratio of the total number of alleles for French and South Tyrolean populations was 2:10, 4:10 and 2:9 for loci Ap2, Ap3 and Ap6, respectively. This would imply that the effective population sizes in South Tyrol have not yet been considerably affected by the fragmentation of habitats.

Contrary to the previous populations, almost complete absence of genetic diversity was found in populations KBL and GIB. Although such a result could emerge from the small number of individuals being analysed from these populations we believe that this finding reflects a recent bottleneck. Only a small number of specimens could be caught from these populations during sampling trials in summer 2003. However, during field studies in the year 2001 no single crayfish was observed at these localities (FÜREDER *et al.*, 2003). Since the patterns of analysed microsatellite loci are exactly the same for all individuals in both populations, we presume that these two brooks were re-stocked quite recently with hatchery reared specimens originating from the same genetic pool, most probably from the HYP population or from another population, which had gone extinct.

The only Austrian population included in this study also lacked genetic variability. Such a result was not surprising, since for the PLS population we expected to see the results of a founder effect. FÜREDER and MACHINO (1995) reported the story given by elder residents about the human introduction of white-clawed crayfish to the lake at the beginning of the 20th century. It seems that these crayfish do not suffer from inbreeding depression as a very large and dense population has developed. Based on mtDNA we can definitely reject the possibility of a French or Carinthian origin of *A. pallipes* in PLS (BARIC *et al.*, 2005). An Italian origin seems very likely, but it is not possible to localise its exact provenience.

In the present study we showed that analyses of population structure and genetic variability on a small geographical scale largely benefit from the use of a combination of different molecular markers. Significant structure among some of the surveyed populations could only be observed after analyzing microsatellite DNA. Nevertheless, mtDNA proved to be a valuable tool in resolving the systematic position of *A. pallipes* from South Tyrol (see BARIC *et al.*, 2005).

Based on our results, we can make management recommendations for the remaining crayfish populations. As we cannot exclude the possibility of long-term genetic isolation of populations, we propose to treat each of the four populations ANG, RIT, KRB and HYP as unique gene pools that warrant individual recognition for conservation and management. All possible measures should be taken to protect, improve and enlarge their habitats in order to prevent a decrease of effective population size and loss of genetic variability. Additionally, brook-specific supportive breeding would contribute to maintain genetic diversity and integrity of populations. If possible, connections among streams should be established to promote gene flow among populations. However, mixing of populations by translocation in order to increase genetic variability should be avoided, since populations could be adapted to local environmental conditions and out breeding could have adverse effects.

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