FIRST ANALYSIS OF GENETIC VARIABILITY IN CARINTHIAN POPULATIONS OF THE WHITE-CLAWED CRAYFISH *AUSTROPOTAMOBIUS PALLIPES*

S. BARIC (1)*, A. HÖLLRIGL (1), L. FÜREDER (2), J. PETUTSCHNIG (3), J. DALLA VIA (1)

(1) Research Centre for Agriculture and Forestry Laimburg. Laimburg 6, 39040 Auer/Ora (BZ), Italy.
E-Mail: sanja.baric@provinz.bz.it

(2) Institute of Zoology and Limnology. University of Innsbruck, Technikerstr. 25, 6020 Innsbruck, Austria.

(3) EB&P - Umweltbüro Klagenfurt GmbH. Bahnhofstr. 39, 9020 Klagenfurt, Austria.

* To whom correspondence should be addressed.

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ABSTRACT

Several authors have speculated about the nativeness of the white-clawed crayfish (*Austropotamobius pallipes*) in the Austrian province of Carinthia, since this is the only occurrence of the species within the Danube river system. Phylogeographic analyses based on mitochondrial DNA sequences showed that Carinthian *A. pallipes* are closely related to specimens from Slovenia, Croatia and north-eastern Italy. These analyses additionally pointed to an absence of variability among populations in Carinthia. In the present study a larger number of individuals sampled from three different brooks in western Carinthia was investigated by analysing sequences of the mtDNA cytochrome c oxidase gene (COI), microsatellite DNA and microsatellite length variation in the nuclear ribosomal DNA internal transcribed spacer region (ITS1). Not only COI sequences revealed low degrees of genetic variability, but also microsatellite loci displayed low allelic diversity and low heterozygosity. Analysis of ITS1 microsatellite length variation in turn showed high levels of intragenomic variability, since single individuals manifested up to seven distinct fragments. Microsatellite data and ITS1 microsatellite length variation analyses also revealed significant differentiation among some population pairs. Since genetic divergence was mainly based on differences in allele frequencies and not on the occurrence of alternative alleles, we assume that Carinthian populations became isolated rather recently and that the occurrence of this species in this region is more likely to be a result of human introduction than of natural postglacial colonisation.

Key-words: *Austropotamobius pallipes*, mitochondrial DNA sequences, 16S rRNA gene, cytochrome c oxidase, microsatellite DNA, ITS1 microsatellite length variation.

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PREMIÈRE ANALYSE DE LA VARIABILITÉ GÉNÉTIQUE DES POPULATIONS D'ÉCREVISSE À PATTES BLANCHES *AUSTROPOTAMOBIUS PALLIPES* EN CARINTHIE

RÉSUMÉ

Plusieurs auteurs ont spéculé sur l'origine de l'écrevisse à pattes blanches (*Austropotamobius pallipes*) de la province autrichienne de Carinthie, parce que c'est la seule référence à la présence de l'espèce dans le bassin du Danube. Les analyses phylogéographiques basées sur des séquences de l'ADN mitochondrial montrent que *A. pallipes* de Carinthie est étroitement liée aux spécimens de Slovénie, Croatie et du nord-est de l'Italie. De plus, ces analyses mettent en évidence l'absence de variabilité au sein de populations de Carinthie. Dans la présente étude un plus grand nombre d'individus prélevés dans trois ruisseaux différents de l'ouest de la Carinthie a été étudié par analyse des séquences du gène mitochondrial cytochrome c oxidase (COI), d'ADN microsatellite et des variations de la longueur des microsatellites au niveau des régions intergéniques transcrites (ITS1) internes des ribosomes nucléaires. Non seulement les séquences du COI révèlent des faibles niveaux de variabilité génétique, mais également les loci microsatellites montrent une faible diversité allélique ainsi qu'une faible hétérozygotie. Par contre les analyses des variations des longueurs des microsatellites ITS1 ont montré des hauts niveaux de variabilité intragénomique, puisque jusqu'à sept fragments distincts ont été révélés pour un seul individu. Les analyses des données microsatellites et des variations de la longueur des microsatellites ITS1 révèlent également une différence significative pour certaines paires de populations. Puisque la divergence génétique est principalement basée sur les différences des fréquences alléliques et non sur les occurrences des allèles alternatifs, nous pouvons affirmer que les populations de Carinthie ont été isolées récemment et que la présence de cette espèce dans cette région est plutôt la conséquence due d'une introduction humaine que d'une colonisation naturelle postglaciaire.

Mots-clés : *Austropotamobius pallipes*, séquences d'ADN mitochondrial, gène 16S rRNA, cytochrome c oxidase, ADN microsatellite, variation de longueur des ITS1 microsatellites.

INTRODUCTION

The white-clawed crayfish *Austropotamobius pallipes* has its main distribution range in Southern and Western European waters. The discovery of this crayfish species in the late 1970ies in a stream of the Danube drainage basin was thus considered a particularity (ALBRECHT, 1981). Since then, in the Austrian province of Carinthia 21 localities inhabited by *A. pallipes* were found in the Gitsch Valley, the Upper Gail Valley and the Upper Drave Valley (MACHINO, 1997; PETUTSCHNIG, 2001a). However, the nativeness of this species has consistently been put into question (MACHINO *et al.*, 2004).

The phylogenetic status of the Carinthian white-clawed crayfish populations was assessed in a previous study by using partial DNA sequences of the mitochondrial large ribosomal subunit (BARIC *et al.*, 2005b). All investigated specimens from Carinthia displayed a single 16S haplotype which clustered in the *A. italicus carsicus* clade defined by GRANDJEAN *et al.* (2000; 2002). Another phylogeographic study concerning the entire genus *Austropotamobius* was based on sequences of the more variable mitochondrial cytochrome c oxidase (COI) gene (TRONTELJ *et al.*, 2005). This study included 11 specimens from eight localities in Carinthia and revealed two distinct COI haplotypes, which were closely related to haplotypes from north-eastern Italy, western Slovenia and Croatia.

The white-clawed crayfish is included in the Red List of threatened and endangered species in Carinthia (PETUTSCHNIG, 1999). For this reason an action plan was elaborated and conservation measures have been carried out (PETUTSCHNIG, 2001b). These include protection and improvement of habitat of existing crayfish populations, renaturation of suitable historical habitats as well as breeding and reintroduction programs. However, decisions about conservation and management could be inefficient without prior understanding of not only the phylogenetic status but also the genetic variability and structure of individual populations. Thus, the latter question was addressed in this study. We chose to investigate three populations of *A. pallipes* covering all parts of its distribution range in Carinthia by applying tools such as sequences of the COI gene, microsatellite DNA and analysis of microsatellite length variation in the nuclear ribosomal DNA internal transcribed spacer (ITS1) region.

MATERIALS AND METHODS

Sampling

Specimens of *A. pallipes* were sampled from three localities in Carinthia (Austria) during autumn in 2003: Gitsch Valley (1), Upper Drave Valley (2) and Upper Gail Valley (3) (see Table 1 and Figure 1). The three sampling sites were located at geographically separated slow-flowing meadow streams, which were partially regulated through agricultural structuring measures. The crayfish from the Gitsch Valley were collected near the Stoffelbauer springs. The samples from the Upper Drave Valley were taken in a meadow stream nearby the village of Armlach, while in the Upper Gail Valley crayfish were sampled in the meadow stream beyond Reisach. A non-destructive sampling procedure was employed by taking the third pereiopod from each individual and preserving each leg separately in absolute ethanol. After sampling all animals were released at the sampling site.

Molecular techniques

COI sequencing

Total nucleic acid was isolated from muscle tissue using a standard chloroform: isoamyl alcohol (24:1) and isopropanol extraction procedure. From 29 specimens an

Table I

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

Tableau I

Sites échantillonnés et nombre d'individus analysés pour chaque population d'*A. pallipes* en Carinthie.

			No of analysed specimens		
No	Sampling site	Site code	COI sequences	3 variable MS loci	ITS microsatellite
1	Gitsch Valley	GIT	10	30	29
2	Upper Drave Valley	DRA	10	29	26
3	Upper Gail Valley	GAI	9	30	30
		Total:	29	89	85

MS... microsatellite.

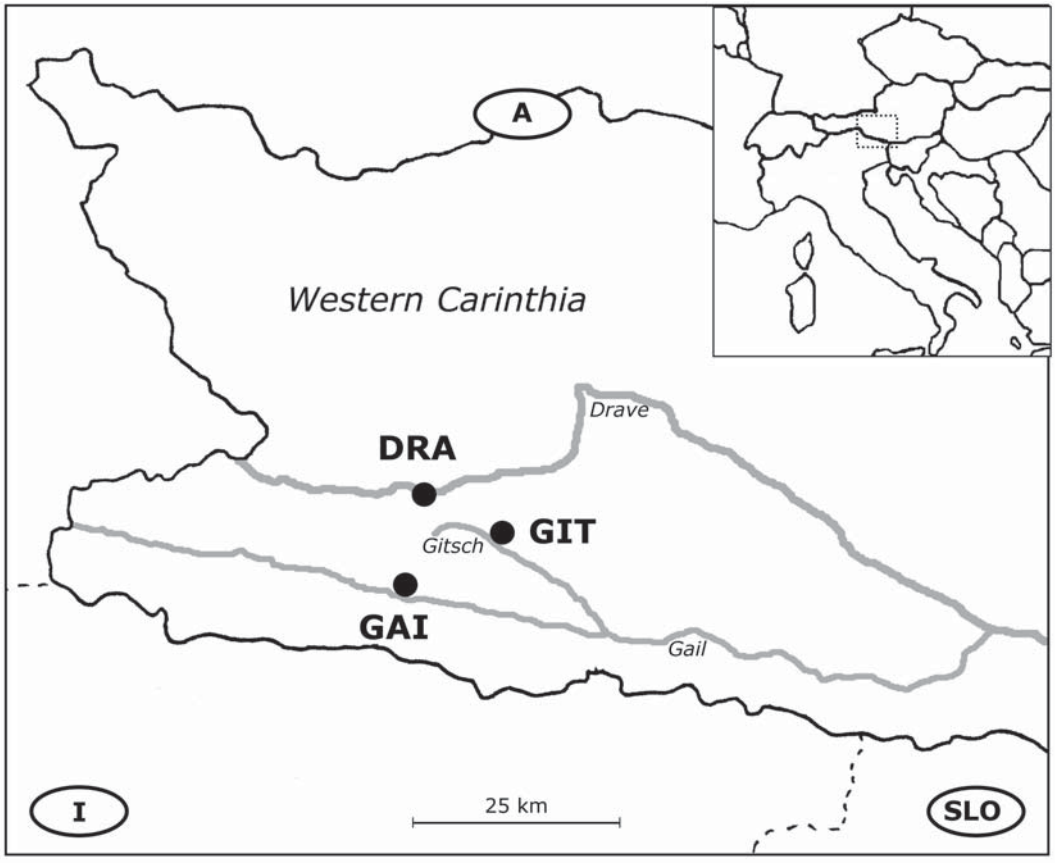

Figure 1

Map of western Carinthia (Austria) with sampling sites for the white-clawed crayfish: GIT (Gitsch Valley, Stoffelbauer springs), DRA (Upper Drave Valley, Armlach) and GAI (Upper Gail Valley, Reisach).

Figure 1

Carte de la Carinthie de l'ouest (Autriche) avec les sites échantillonnés en écrevisses à pattes blanches: GIT (Vallée de Gitsch, sources de Stoffelbauer), DRA (vallées de Upper Drave, Armlach) et GAI (vallées de Upper Gail, Reisach).

approximately 1,000-bp segment of the mtDNA cytochrome c oxidase subunit I (COI) was amplified with primers Ap-COI-SB-F (5'-GGGACGATCAAATTTATAATGTAGTAGTT-3') and Ap-COI-LB-R (5'-AAACAAAGGAAATCCATGAAC-3'; BARIC *et al.,* 2005a). Fifty microliter reaction volumes contained 1 µM of each primer, 200 µM dNTP-Mix (Eppendorf, Germany), 2 mM $MgCl_2$, 1 × GeneAmp PCR Buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA), and 2 µl (25-100 ng) DNA template. Amplification reactions were run on the Mastercycler Gradient (Eppendorf, Germany) or GeneAmp PCR System 2700 (Applied Biosystems, USA) under the following conditions: 10 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 mentioned above, two newly designed internal sequencing primers, Ap-COI-SB-R (5'-TTGATATAAAACCGGGTCTCCT-3') and Ap-COI-SB-R* (5'-AGGAGACCCGGTTTTATATCAA-3'), were used. 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).

Microsatellite analysis

In a preliminary study seven microsatellite loci, Ap1, Ap2, Ap3, Ap4, Ap5, Ap6 and Ap7 developed by GOUIN *et al*. (2000; 2002) and locus Ait1 developed in our laboratory (GenBank accession number DQ250049; Ait1-F: 5'-CGTTAGTGCTTGTACTGTATCT-3', Ait1-R: 5'-TACGAGCGTGTATCTCGT-3') were assessed to be used with Carinthian populations of *A. pallipes.* In a second step 89 specimens were analysed at the three variable loci Ap3, Ap6 and Ait1 (Table 1). Ten microliter amplification reaction volumes contained 0.2 µM (Ap3), 0.4 µM (Ap6) or 1 µM (Ait1) of each primer, 200 µM dNTP-Mix (Eppendorf), 1 × HotMaster Taq Buffer with Mg^{2+} (2.5 mM Mg^{2+}), 0.5 U HotMaster Taq DNA polymerase (Eppendorf) and 2 µl (25-100 ng) 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 (Ap3 and Ap6) or 55°C (Ait1) and 60 s at 65°C, and a final extension of 10 min at 65°C. Each forward primer was fluorescently labelled with a Beckman WellRED dye and samples were run with the internal CEQ DNA Size Standard – 400 (Beckman Coulter) and visualised 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.

ITS1 length variation

In addition to the microsatellite loci developed for *A. pallipes*, length variation in the rDNA ITS1 region was assessed as described by EDSMAN *et al*. (2002). PCR was performed in 10 µl volumes containing 1 µM of each primer Asa1F and Asa1R (EDSMAN *et al.*, 2002), 200 µM dNTP-Mix (Eppendorf, Germany), 1.5 mM $MgCl_2$, 1 × GeneAmp PCR Buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 0.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA) and 2 µl (25-100 ng) DNA template. PCR conditions were set to 10 min at 94°C followed by 38 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, and a final extension step at 72°C for 10 min. Primer Asa1F was fluorescently labelled with Cy5 and fragment lengths were analysed as described above. Fragment patterns were examined by eye and only clear, reproducible bands were scored as alleles, while weak fragments and stutter band-like patterns were not scored (see Figure 2).

Since fragments found for *A. pallipes* were approximately 150 bp longer than that found for *Astacus astacus*, purified PCR products from two specimens were cloned by using the pGEM-T Easy VectorSystem II (Promega, Madison, USA) following the manufacturer's protocol. Plasmids were purified with the Eppendorf Perfectprep Plasmid Isolation Kit (Eppendorf) and inserts were sequenced by using Primer 20mer (Novagen, EMD Biosciences, Germany) and Sequencing Primer M13-47 (Beckman Coulter) according to the procedure described before.

Data analyses

Partial sequences of the COI gene were aligned and controlled using the computer program SEQUENCHER Version 4.2 for Windows (Gene Codes Corporation, Ann Arbor, USA). PAUP* version 4.0 Beta (SWOFFORD, 2002) was used to calculate pairwise distances among haplotypes obtained in this study and sequences of *A. pallipes* from Carinthia already published in NCBI GenBank (http://www.ncbi.nlm.nih.gov/).

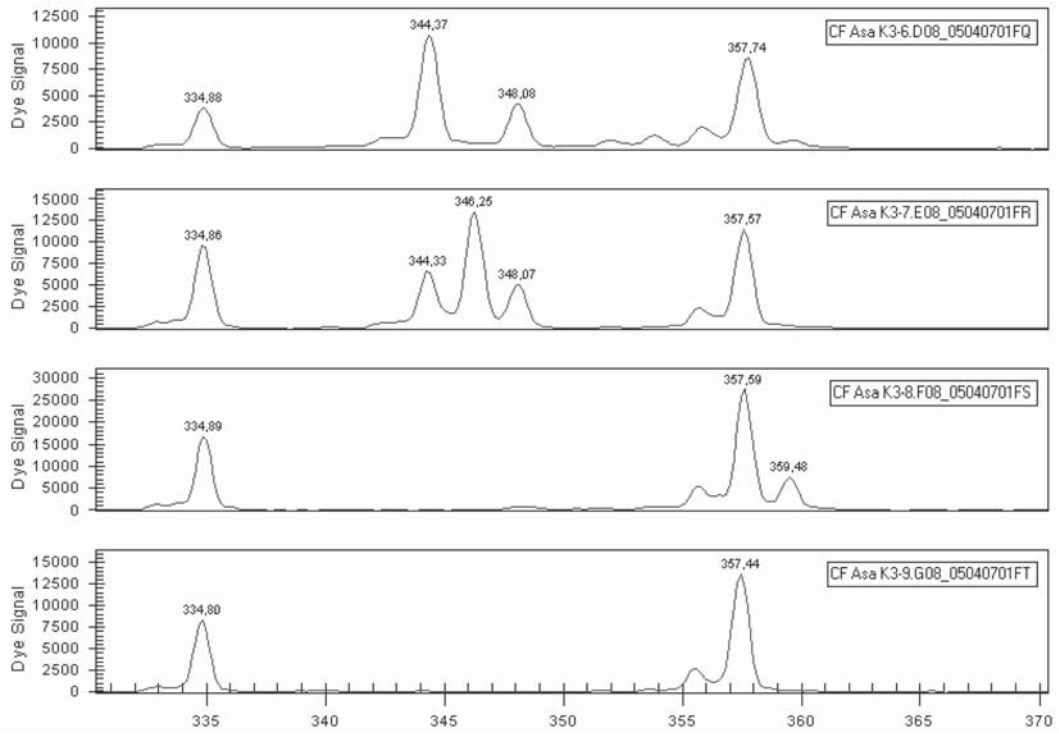

Figure 2

Electropherograms of four samples showing different fragment patterns for the rDNA ITS1 region. Only clear, reproducible peaks were scored as alleles (size marks), while weak fragments and stutter band-like patterns were not scored.

Figure 2

Électrophorégrammes de quatre échantillons montrant des profils différents pour la région rDNA ITS1. Seuls les pics bien définis et reproductibles ont été recensés en tant qu'allèles (tailles indiquées), alors que les profils montrant des fragments faibles et mal définis n'ont pas été retenus.

Genetic variability of the three microsatellite loci was estimated for each population in terms of allele and genotype 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). Tests for significant heterozygote deficit and excess based on the Markov chain method (GUO and THOMPSON, 1992) were performed with the software GENEPOP version 3.3 (RAYMOND and ROUSSET, 1995). The same software was used to test for genotypic linkage disequilibrium for all pairs of loci in each population as well as across populations in order to verify that there was no association among genotypes at different loci. Exact probability tests for genotypic and genic differentiation among all pairs of populations were conducted with GENEPOP, while ARLEQUIN was used to estimate 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). ITS1 microsatellite data were analysed by performing the Population Divergence Test implemented in the computer program PDT (FARRIS, 2000; EDSMAN *et al.*, 2002) at the Swedish Museum of Natural History, Stockholm, Sweden.

RESULTS

Partial sequences of the COI gene were obtained from 29 specimens and the length of the alignment was 943 nucleotide positions. A single haplotype was found in 28 individuals sampled from all three populations. Only one specimen from population GAI carried a second haplotype which differed by one base pair substitution. Our sequences could be aligned with 541 bp of the GenBank sequences AY667112 (found at seven different localities in Carinthia) and AY667118 (found at one locality in Carinthia) (TRONTELJ *et al.*, 2005), and with 414 bp of the GenBank sequence AY121117. After excluding the last nucleotide position of sequences AY667112 and AY667118 (which differed most probably due to a sequencing artefact) we found that the most common haplotype identified in the present study was identical to sequences AY667112 and AY121117.

In the investigated populations of *A. pallipes* three microsatellite loci, Ap3, Ap6 and Ait1, were found to be polymorphic, each with two alleles (Table 2). Locus Ap1 turned out to be monomorphic after analysing 88 specimens from all three populations. The other microsatellite loci tested either did not amplify (Ap2, Ap5, Ap7) or did not yield scorable results (Ap4).

The average observed heterozygosity across all three variable loci and populations amounted to 0.35 (± 0.182). After sequential Bonferroni correction significant deviation from expected Hardy-Weinberg proportions was found at locus Ap6 for all three populations (Table 3). This could have occurred due to the presence of a null allele or because of the dropout of larger alleles that were outcompeted by short alleles during PCR. The exact probability test for heterozygote deficit across all loci showed a heterozygote deficit in the DRA population, while the test across populations indicated heterozygote deficit at locus Ap6 (Table 3). However, for population DRA the test across loci could have been biased by the extreme deviation of locus Ap6. None of the populations and loci had a heterozygote

Table II

Allele and genotype frequencies at three microsatellite loci in Carinthian populations of *A. pallipes*

Tableau II

Fréquences alléliques et génotypiques de trois loci microsatellite dans les populations d'*A. pallipes* en Carinthie.

	Allele frequencies		Genotype frequencies		
Locus Ap3	**149**	**151**	**149/149**	**149/151**	**151/151**
GIT	0.62	0.38	0.38	0.48	0.14
DRA	0.78	0.22	0.66	0.24	0.10
GAI	0.47	0.53	0.27	0.40	0.33
Locus Ap6	**370**	**378**	**370/370**	**370/378**	**378/378**
GIT	0.58	0.42	0.48	0.20	0.32
DRA	0.72	0.28	0.70	0.04	0.26
GAI	0.57	0.43	0.46	0.21	0.32
Locus Ait1	**276**	**278**	**276/276**	**276/278**	**278/278**
GIT	0.38	0.62	0.10	0.55	0.34
DRA	0.40	0.60	0.17	0.45	0.38
GAI	0.68	0.32	0.40	0.57	0.03

Table III

Summary table of variation detected at three microsatellite loci in three Carinthian *A. pallipes* populations. Observed (H_O) and expected (H_E) heterozygosity as well as results of global Hardy-Weinberg exact probability tests for heterozygote deficit and excess are given. Asterisks indicate significant deviation from Hardy-Weinberg equilibrium or statistical significance of P-values at the 5% (*), 1% (**) and 0.1% (***) level after sequential Bonferroni correction.

Tableau III

Tableau résumant les variations détectées aux trois loci microsatellites dans les trois populations d'*A. pallipes* échantillonnées en Carinthie. Hétérozygotie observé (H_0) et attendue (H_E) et résultats des tests de probabilité exacte d'équilibre de Hardy-Weinberg en ce qui concerne le déficit ou l'excès d'hétérozygotes. Les astérisques indique les déviations significatives de l'équilibre de Hardy-Weinberg ou le degré de significativité des valeurs de P à 5% (*), 1% (**) et 0.1% (***) après correction séquentielle de Bonferroni.

		Population		
		GIT	**DRA**	**GAI**
Locus Ap3	H_O	0.483	0.241	0.400
	H_E	0.492	0.381	0.506
Locus Ap6	H_O	0.200	0.043	0.214
	H_E	0.551*	0.571***	0.551*
Locus Ait1	H_O	0.552	0.448	0.567
	H_E	0.492	0.500	0.440
Mean H_O		0.412	0.244	0.394
Mean H_E		0.512	0.484	0.499
Heterozygote deficit	**P**	0.1649	0.0002**	0.0767
	SE	0.0004	0.0000	0.0003
Heterozygote excess	**P**	0.8347	0.9998	0.9233
	SE	0.0004	0.0000	0.0003
		Locus		
		Ap3	**Ap6**	**Ait1**
Heterozygote deficit	**P**	0.0554	0.0000***	0.9060
	SE	0.0002	0.0000	0.0003
Heterozygote excess	**P**	0.9448	1.0000	0.0997
	SE	0.0002	0.0000	0.0003

excess. The exact test for genotypic linkage disequilibrium did not indicate significant association of genotypes for any pair of loci (P-values ranged from 0.155 to 0.908).

Exact tests of population differentiation across all loci revealed significant differences in both, allele and genotype frequency between population pairs GAI and GIT, and GAI and

DRA (Table 4). Population pair GIT and DRA did not show significant genotypic or allelic differentiation. Congruent with the results of the exact tests of population differentiation, pairwise F_{ST} values were significantly different from zero for population pairs GAI and GIT, and GAI and DRA, while no significant difference was found for population pair GIT and DRA. Pairwise F_{ST} estimates ranged from 0.013 to 0.116, with a mean value of 0.064 (± 0.0513; Table 4).

Analysis of the microsatellite length variation in the ITS1 region revealed eight different fragments in *A. pallipes* with a length from 334 to 360 bp. Intragenomic variation was rather pronounced since single individuals were found to carry two to seven distinct fragments. Reproducibility of fragment patterns could be confirmed by amplifying and electrophoresing approximately 10% of the samples twice independently. Six of the fragments were distributed in all three populations, while fragment 352 was found at low frequencies in populations GIT and DRA, and fragment 346 was found in only one individual in population GAI. Across all populations 22 different fragment patterns were observed. Three of these fragment patterns were distributed in all populations, although at different frequencies. Populations GIT and DRA shared four additional fragment patterns, while population GAI showed the highest number of unique fragment patterns (Table 5). The population divergence test performed with the ITS1 microsatellite dataset resulted in significant differences between all three population pairs surveyed for Carinthia (Table 4).

The results of the cloning and sequencing of the ITS1 segment confirmed that we were indeed scoring a microsatellite in *A. pallipes* by using primers Asa1F and Asa1R,

Table IV

Results of pairwise population comparisons using data from three variable microsatellite loci and the rDNA ITS microsatellite. Population pairwise F_{ST} estimates with levels of significance after sequential Bonferroni correction are given as well as the significance levels for the exact tests for population differentiation (genic and genotypic). P-values are given for the population divergence test performed with the ITS microsatellite dataset.

Tableau IV

Résultats des comparaisons par paire de populations à partir des données obtenues avec trois loci microsatellites variables et le rDNA ITS microsatellite. L'estimation des F_{ST} par paire de populations et leur niveau de significativité après correction séquentielle de Bonferroni correction sont données ainsi les niveaux de significativité relatifs aux tests exacts pour la différenciation des populations (génétique et génotypique). Les valeurs de P sont indiquées pour le test de divergence des populations calculé avec les données concernant le ITS microsatellite.

	Three variable microsatellite loci			ITS microsatellite
		Exact test for population differentiation		
Population pair	F_{ST} values	Genic	Genotypic	Population divergence test (P-value)
GIT – DRA	0.0134^{NS}	NS	NS	0.0088
GIT – GAI	0.0627*	*	**	0.0001
DRA – GAI	0.1160**	**	***	0.0000

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

Table V

Distribution of different rDNA ITS1 fragment patterns in three Carinthian *A. pallipes* populations. Number sequences indicate different fragment lengths in bp. Unique fragment patterns found only in single populations are given in italics, while these being distributed in all three populations are shaded in grey.

Tableau V

Distribution des différents profils rDNA ITS1 chez trois populations d'*A. pallipes* de Carinthie. Le nombre de séquences indique différentes longueurs de fragment en pb. Les profils uniques trouvés dans une seule population sont en italiques alors que ceux distribués dans les trois populations sont en grisé.

	Population		
Fragment pattern	**GIT**	**DRA**	**GAI**
334/344/346/348/358			*1*
334/344/348	1	1	
334/344/348/350/352/358		*3*	
334/344/348/350/352/358/360	2	5	
334/344/348/350/358	5	2	1
334/344/348/350/358/360	6	5	
334/344/348/350/360	*1*		
334/344/348/358	5	4	9
334/344/348/358/360		1	4
334/344/358			*3*
334/344/358/360			*2*
334/348/350/358	*4*		
334/348/350/358/360	*1*		
334/348/358	1		1
334/348/358/360			*1*
334/358			*2*
334/358/360			*2*
344/348/350/352/358/360		*1*	
344/348/350/358/360	2	1	
344/350/352/358/360		*1*	
344/350/358/360	1	2	2
344/358			2
N fragment patterns per population:	11	11	12

which were previously believed to be specific for the noble crayfish *Astacus astacus*. Sequences were obtained from six clones corresponding to four different fragments with lengths of 348, 350, 352 and 360 bp. The flanking region before the dinucleotide GA-repeat was 229 bp long and the repeat motif was $(GA)_{12\text{-}17}AAGA(AA)(GA)_4$. Sequences of

the four fragments are available from GenBank under the accession numbers DQ250050-DQ250053.

DISCUSSION

Both mitochondrial DNA sequences and microsatellite DNA data from Carinthian white-clawed crayfish populations displayed low degrees of genetic variability. Sequence analyses of the mtDNA COI gene revealed one common haplotype, which was distributed in all the three populations surveyed. The same haplotype was found by TRONTELJ *et al*. (2005) at seven different localities in western Carinthia. Only in one population (GAI) we found a single specimen with a distinct haplotype differing by one point mutation. However, it is unclear whether it represents an ancient haplotype, which became rare by the stochastic process of lineage sorting or it has arisen by mutation in this particular population. In comparison to Carinthia much higher haplotype diversity at the COI gene was found in neighbouring regions of Slovenia, Croatia and north-eastern Italy (TRONTELJ *et al*., 2005). On the other hand, populations in the northern and western distribution areas of *A. pallipes* were observed to be genetically more uniform. While it is not that surprising to find low levels of genetic variability by analysing sequences of the relatively slowly evolving and maternally inherited mtDNA (NGUYEN and AUSTIN, 2004), it is remarkable that also microsatellite DNA data revealed extremely low allelic diversity. At each of the three polymorphic microsatellite loci employed in the present work only two different alleles were found. By comparison, a previous study investigating populations of *A. pallipes* in South Tyrol (Italy) found a total number of ten and nine different alleles at loci Ap3 and Ap6 (BARIC *et al*., 2005a). Similarly, overall heterozygosity was found to be lower in Carinthian (H_O = 0.35) than in South Tyrolean populations (H_O = 0.53), while it was comparable to that observed in a single brook in France (H_O = 0.38; GOUIN *et al*., 2002).

Since its (re)discovery in 1977 several authors have speculated about the origin of the white-clawed crayfish in western Carinthia (ALBRECHT, 1981; LARGIADÈR *et al.*, 2000; MACHINO, 1997; MACHINO *et al.*, 2004; MACHINO and FÜREDER, 1996), because this is the only incidence of the species within the Danube river system. After thoroughly studying historical records MACHINO *et al*. (2004) found that documents mentioning *A. pallipes* in Carinthia were scarce and the first record about the species appeared on the turn from the 19th to the 20th century. The same authors pointed out that crayfish translocation and introduction was quite a common human activity in Europe. Therefore, it seems to be probable that the occurrence of the white-clawed crayfish in Carinthia is due to anthropogenic introduction. This assumption would be supported by the low genetic variation found in the present study. Translocation usually involves a limited number of individuals and leads to severe bottlenecks: the consequences in founder populations are decrease of genetic variability and effective population size (LE PAGE *et al.*, 2000; NEI *et al.*, 1975). Hence, such populations are more strongly affected by the action of random processes, like genetic drift, which can promote morphological differentiation. Diversification of morphological traits could in addition be caused by selective forces acting in the new habitat (GRANT *et al.*, 2001; RASNER *et al.*, 2004). Indeed, for the white-clawed crayfish in Carinthia some morphological peculiarities were observed that led ALBRECHT (1981) to describe an independent variety *A. pallipes* var. *carinthiacus*.

A combined effect of random genetic drift and inbreeding might also be responsible for detecting genetic differentiation among populations. The analysis of microsatellite loci revealed significant differentiation among population pairs GAI-GIT and GAI-DRA. Since each of the three populations carried exactly the same alleles, genetic distinction is exclusively due to differences in allele frequencies and not caused by the preservation of alternative alleles. Finding the same allelic patterns across all three loci and all three populations either implies extensive gene flow before the actual fragmentation of habitats or a relatively recent origin from a source population with little genetic variability. GOUIN

et al. (2002) studied gene flow in *A. pallipes* and demonstrated that, in the absence of a barrier, the species is able to spread over remarkable distances of favourable habitat. However, the study was performed in a single brook and distances among study sites were much smaller than that in the present work. Indeed, in South Tyrol considerable population differentiation was found on a relatively small geographical scale together with moderate degrees of genetic variability within populations (BARIC *et al.,* 2005a). It is believed that this region was naturally colonised by the white-clawed crayfish after the last glaciation about 15,000-10,000 years ago. For this reason, the absence of significant population differentiation among the Carinthian populations GIT and DRA, which are additionally geographically separated by a mountain chain, can hardly be explained by ongoing gene flow. We thus believe that the occurrence of *A. pallipes* in Carinthia is more likely to be a result of human introduction than of natural postglacial colonisation.

A surprising result of this study was the fact that a genetic marker, originally developed for *Astacus astacus*, could directly be employed for population genetic analysis of the white-clawed crayfish. EDSMAN *et al.* (2002) designed a primer combination which amplifies a segment of the nuclear ribosomal DNA ITS1 region containing a length-variable microsatellite repeat. The authors concluded that the primers were specific for *A. astacus* after acquiring no amplicon with a set of DNA isolates from different crayfish species also including the closely related *Astacus leptodactylus*. However, we demonstrate that the primers work as well with at least one 16S clade of the *A. pallipes* species complex. The substantial difference was found to be the fragment lengths: while in *A. astacus* fragments ranged form 162 to 210 bp, in *A. pallipes* fragments were much longer, ranging from 334 to 360 bp. Analysis of ITS1 microsatellite length variation additionally revealed remarkably high degrees of intragenomic variability in the white-clawed crayfish as single individuals manifested up to seven distinct fragments. This phenomenon can be explained by the repetitive nature of the ITS, being part of the eukaryotic ribosomal DNA array. Normally, concerted evolution leads to homogenisation between all repeats of the same multigene family (ELDER and TURNER, 1995; LIAO, 1999), but in the case that it is inefficient, intraindividual heteroplasmy can occur (EDSMAN *et al.*, 2002; HARRIS and CRANDALL, 2000; VOGLER and DESALLE, 1994). For several freshwater crayfish species of the genera *Procambarus* and *Orconectes* the amount of intragenomic variation within ITS1 and ITS2 was shown to even exceed the variation between different species (HARRIS and CRANDALL, 2000). For this reason multicopy genes should be used with caution in phylogenetic studies. Nevertheless, EDSMAN *et al.* (2002) pointed out that examination of microsatellite length variation in the ITS1 region, combined with a special statistical test (FARRIS, 2002), could be a practical tool for assessing divergence between populations of the noble crayfish. In fact, the ITS1 microsatellite dataset revealed significant differences among all three Carinthian populations of the white-clawed crayfish. It might be speculated that certain multicopy gene alleles could be removed or diminished in their frequency more rapidly by stochastic processes than alleles of classical microsatellite loci, and therefore this marker exhibits higher degrees of population structure.

At the present state of knowledge we propose that the Upper Gail Valley should be treated as a separate management unit since the white-clawed crayfish from this area displayed the highest degree of genetic divergence and also differed in some morphological characters, such as the number of spines in the cervical groove (MACHINO, 1997). In the GAI population also one individual with a distinct mtDNA haplotype and a second individual with a private ITS1 microsatellite fragment were found, as well as a higher number of unique ITS1 genotypes. However, in order to give more profound recommendations for conservation and management, additional populations from this region need to be analysed. Ideally, these analyses should be expanded to include populations from Slovenia, Croatia and north-eastern Italy for finding an answer about the origin of the Carinthian white-clawed crayfish.

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