

**UPDATE OF THE MOLECULAR PHYLOGENY OF
THE *AUSTROPOTAMOBIOUS PALLIPES* SPECIES COMPLEX
BY INCLUDING SPECIMENS FROM SOUTH TYROL (ITALY)
AND CARINTHIA (AUSTRIA)**

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ABSTRACT

The phylogenetic relationships of the *Austropotamobius pallipes* species complex were assessed in several recent genetic studies by analysing sequences of the mitochondrial DNA. Although the Alpine region is known to harbour great extents of morphological and genetic diversity, none of the previous studies included populations from South Tyrol in Northern Italy and Carinthia in Southern Austria. In order to clarify their taxonomic status white-clawed crayfish were sampled from six populations in South Tyrol and three populations in Carinthia. Phylogenetic analyses are based on partial DNA sequences of the mitochondrial DNA 16S rRNA gene, which were supplemented with sequences deposited in GenBank. All surveyed individuals from the six populations in South Tyrol comprised a single haplotype that equalled the already described haplotype S3. This haplotype is distributed in Plansee (Austria) as well as several other localities in Italy and Switzerland, and belongs to the *A. italicus carinthiacus* clade. The investigated specimens from all three Carinthian populations also displayed one single haplotype. However, this haplotype clustered in the *A. italicus carsicus* clade and has not been published before. Our study clearly shows that neither Austrian crayfish populations from Plansee and Carinthia, nor populations from South Tyrol and Carinthia belong to the same phylogenetic lineage.

Key-words: white clawed crayfish, molecular phylogeny, mitochondrial DNA 16S rRNA gene.

**MISE À JOUR DE LA PHYLOGÉNIE MOLÉCULAIRE DU COMPLEXE D'ESPÈCES
AUSTROPOTAMOBIOUS PALLIPES EN INCLUANT DES SPÉCIMENS DU TYROL
DU SUD (ITALIE) ET DE CARINTHIE (AUTRICHE)**

RÉSUMÉ

Les relations phylogénétiques du complexe d'espèces *Austropotamobius pallipes* ont été évaluées dans plusieurs études génétiques récentes par des analyses de

séquences de l'ADN mitochondrial. Bien que les régions alpines soient connues pour héberger une grande diversité morphologique et génétique, aucune des études précédentes n'a inclus des populations du sud Tyrol dans le Nord de l'Italie et de Carinthie en Autriche méridionale. Pour clarifier le statut taxinomique de l'écrevisse à pattes blanches nous avons échantillonné six populations du Tyrol du Sud et trois en Carinthie. Les analyses phylogénétiques sont basées sur des séquences partielles d'ADN du gène mitochondrial codant pour l'ARNr 16S, complétées par des séquences déposées dans GenBank. Tous les individus des six populations du sud Tyrol analysés ont un haplotype simple identique à l'haplotype S3 déjà décrit. Cet haplotype, appartenant au clade de *A. italicus carinthiacus*, est présent dans le Plansee (Autriche) ainsi que dans plusieurs localités italiennes et suisses. Les spécimens provenant des trois populations de Carinthie présentent aussi un seul haplotype. Cependant, cet haplotype clustérisé dans la clade de *A. italicus carsicus* n'a pas été publié auparavant. Notre étude prouve clairement que ni les populations d'écrevisses autrichiennes de Plansee et de Carinthie, ni les populations du Tyrol du Sud et de Carinthie n'appartiennent à la même lignée phylogénétique.

Mots-clés : écrevisses à pattes blanches, phylogénie moléculaire, gène mitochondrial ARNr 16S.

INTRODUCTION

The white-clawed crayfish *Austropotamobius pallipes* has a wide distribution range across Western Europe, covering the Iberian Peninsula, France, Great Britain, Ireland, Switzerland, Austria, Italy and the Adriatic coast of Slovenia and Croatia (LAURENT, 1988). However, over the past decades the number of *A. pallipes* populations has been severely declining and nowadays the white-clawed crayfish represents one of the most endangered freshwater species in Europe. The main reasons for the decline of this species are alteration and destruction of natural habitats, water pollution as well as stocking with alien crayfish and predatory fish species. Besides biotic interactions, allochthonous species can be vectors of the crayfish plague, which was introduced to Europe at the end of the 19th century (HOLDICH and LOWERY, 1988). Since then, numerous outbreaks of the disease have led to the eradication of many local populations. Thus, many countries started implementing protection programs for the white-clawed crayfish that, amongst other measures, include the enhancement of environmental conditions, the protection and improvement of habitat structure, and restocking and reintroduction of the species. Several studies have demonstrated that the latter two actions should never be carried out without a profound knowledge about the taxonomic status and the genetic structure of local populations, as inadequate management schemes could have dramatic consequences (HUGHES *et al.*, 2003; WOLF *et al.*, 2001; RHYMER and SIMBERLOFF, 1996). However, the taxonomy of the white-clawed crayfish based on morphological and meristic criteria is highly controversial proposing (i) one single species, (ii) several varieties or subspecies or even (iii) several species (reviewed in GRANDJEAN *et al.*, 2002b and HOLDICH, 2002). Therefore phylogenetic relationships of the *A. pallipes* species complex were assessed in several recent genetic studies using partial DNA sequences of the mitochondrial large ribosomal subunit (GRANDJEAN and SOUTY-GROSSET, 2000; GRANDJEAN *et al.*, 2000; 2001; 2002a, 2002b; LARGIADÈR *et al.*, 2000). Based on mitochondrial DNA (mtDNA) sequence data in combination with morphological characters, GRANDJEAN *et al.* (2002a, 2002b) proposed a new classification for the *A. pallipes* species complex defining two species, *A. pallipes* and *A. italicus*, and within the latter three subspecies, *A. italicus italicus*, *A. italicus carinthiacus* and *A. italicus carsicus*.

Despite the fact that molecular data displayed pronounced genetic diversity in the Alpine region (LARGIADÈR *et al.*, 2000; LÖRTSCHER *et al.*, 1997; SANTUCCI *et al.*, 1997), since several major European drainage systems originate in this area and mountains

might act as natural barriers to gene flow, none of the previous genetic studies addressed the phylogenetic status of white-clawed crayfish populations from South Tyrol (Northern Italy) and Carinthia (South-Western Austria). As in both of these regions breeding and reintroduction programs are being carried out (FÜREDER *et al.*, 2002; PETUTSCHNIG, 2001), it became highly necessary to clarify the taxonomic status of *A. pallipes* in these two Alpine areas by applying genetic and phylogenetic analyses.

MATERIALS AND METHODS

Specimens of *A. pallipes* were sampled from a total of ten localities in Northern Italy (Autonomous Province of Bozen/Bolzano – South Tyrol) and Austria (counties Tyrol and Carinthia) by hand catches or crayfish traps. From each of the 47 individuals the third pereopod was taken and preserved separately in absolute ethanol. The animals were then released at the sampling locality. Table 1 and Figure 1 give the sampling localities and the number of individuals analysed per site.

Table I

Sample localities and number of individuals (N) included in the present study.

Tableau I

Localités échantillonnées et nombre d'individus (N) inclus dans la présente étude.

No	Sampling site	Site code	N
1	Angelbach ^a	ANG	7
2	Ritscherbach ^a	RIT	10
3	Krebusbach ^a	KRB	10
4	Hyppolithbach ^a	HYP	6
5	Krebsbach Lana ^a	KBL	2
6	Gießbach ^a	GIB	3
7	Plansee ^b	PLS	3
8	Gitschtal ^c	GIT	2
9	Oberdrautal ^c	DRA	2
10	Obergailtal ^c	GAI	2
Total:			47

^a South Tyrol (Italy); ^b Tyrol (Austria); ^c Carinthia (Austria).

Total nucleic acid was isolated from muscle tissue according to the procedure of SAMBROOK *et al.* (1989). An approximately 500 bp-segment of the mtDNA 16S rRNA gene was amplified with primers 1472 and 1471 (CRANDALL and FITZPATRICK, 1996). Ten microliter reaction volumes contained 1 μ M of each primer, 200 μ M dNTP-Mix (GeneCraft, Germany), 1.5 mM MgCl₂, 0.5 U BioTherm DNA polymerase (GeneCraft) and 2 μ l 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. PCR products were separated and visualized on ethidium bromide stained 1.5% agarose gels, and purified using Montage PCR Centrifugal Filter Devices (Millipore, USA). Sequencing was

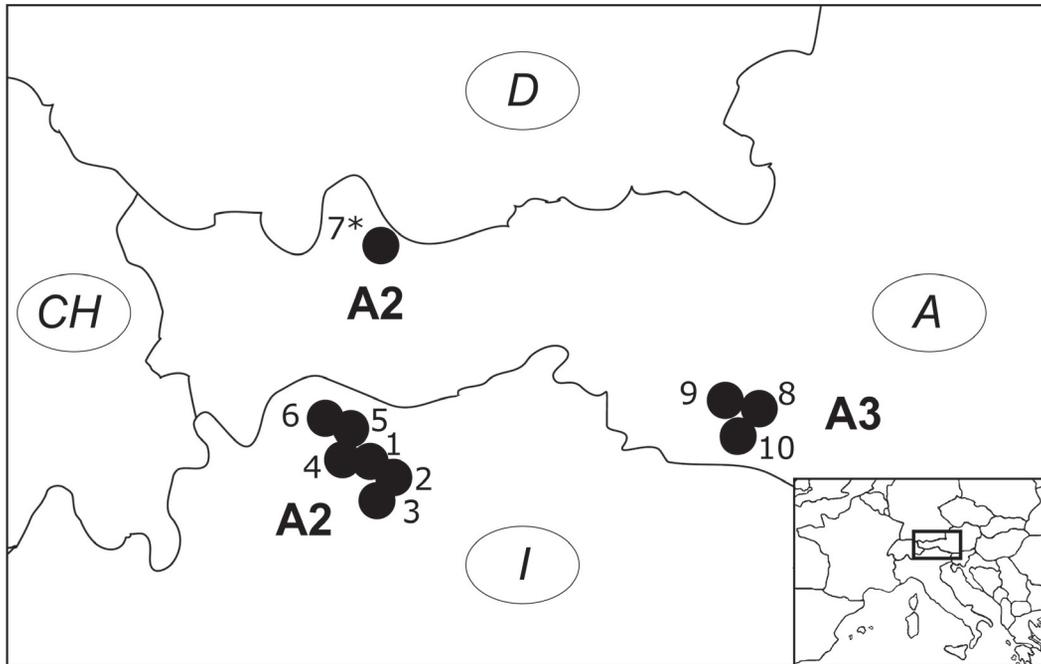


Figure 1

Map showing the geographical localities of populations of the *A. pallipes* species complex analysed in the present study. A2 and A3 indicate the assignment of populations to the 16S mtDNA sub-lineage *A. italicus carinthiacus* and *A. italicus carsicus*, respectively. Locality names are given in Table 1. Population 7 (Plansee; denoted by asterisk) was already analysed in previous works (LARGIADÈR *et al.*, 2000; GRANDJEAN *et al.*, 2002).

Figure 1

Carte montrant les localisations géographiques des populations du complexe d'espèces *A. pallipes* analysé dans la présente étude. A2 et A3 indiquent l'appartenance des populations au sous-lignage respectif *A. italicus carinthiacus* et *A. italicus carsicus* par l'ADNr 16S. Les noms des localités sont donnés dans la Table 1. La population 7 (Plansee ; noté par un astérisque) a déjà été analysée dans une étude antérieure (LARGIADÈR *et al.*, 2000 ; GRANDJEAN *et al.*, 2002).

performed in both directions in 10 µl reaction volumes with the CEQ DTCS Quick Start Kit (Beckman Coulter, USA) following the manufacturer's protocol. Sequencing products were separated and detected on the CEQ 8000 Genetic Analysis System (Beckman Coulter).

The newly obtained sequences were examined by using the software SEQUENCHER Version 4.2 for Windows (Gene Codes Corporation, Ann Arbor, USA). In a second step partial 16S sequences were supplemented with 17 *A. pallipes* sequences from GenBank, which were previously acquired by GRANDJEAN *et al.* (2000) and LARGIADÈR *et al.* (2000), and were compiled by GRANDJEAN *et al.* (2002a, 2002b) for the phylogeny of the *A. pallipes* species complex covering almost its entire geographical range. The accession numbers of these sequences were: AJ242700, AJ242701, AJ242704, AJ242706, AJ242708, AJ242709, AJ242711, AF237590, AF237593, AF237595, AF237596, AF237597, AF237601, AF237603, AF237604, AF237605 and AF237609. One additional sequence of *A. torrentium* (accession number: AJ242699) was included to be used as outgroup.

Sequences were aligned by eye. PAUP* version 4.0 Beta (SWOFFORD, 2002) was used to compute a matrix of pairwise distances in order to identify identical sequences and assign them to a particular haplotype. The reduced dataset was then employed for phylogenetic inferences by using maximum parsimony (MP) and neighbour joining (NJ) as implemented in PAUP* (SWOFFORD, 2002). MP analyses were performed using the heuristic search option based on branch swapping with tree bisection-reconnection and random addition of taxa. All characters had equal weight and gaps were treated as the fifth base. NJ analyses were done applying the HKY85 model (HASEGAWA *et al.*, 1985) and treating gaps as missing data. Confidence for the resulting groups was assessed using the bootstrap procedure with 1,000 replicates (FELSENSTEIN, 1985).

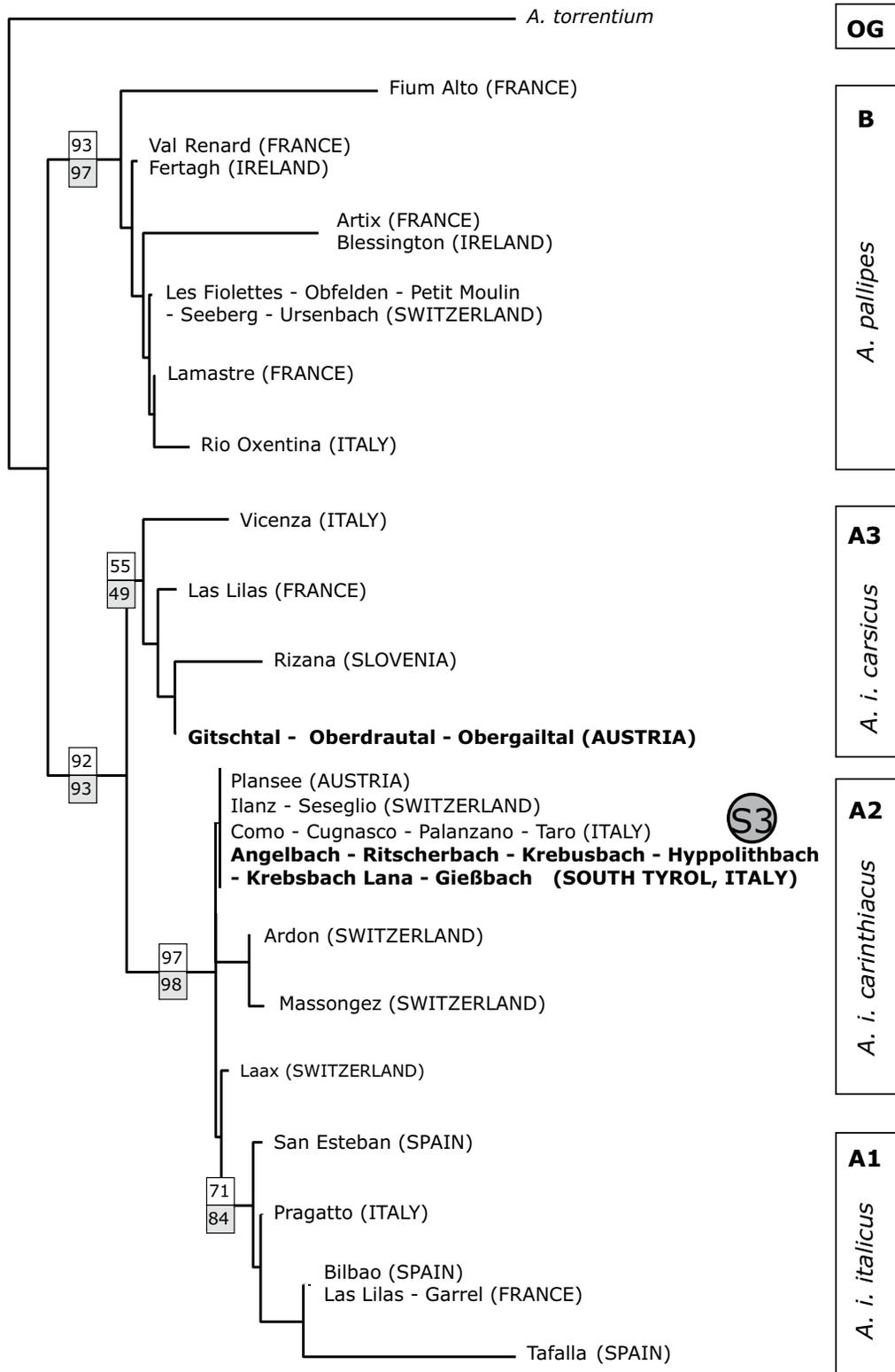
RESULTS

The matrix of absolute pairwise distances, in which gaps were treated as missing data, did not reveal any base pair substitution among individuals from the six populations in South Tyrol and the Plansee population (Tyrol, Austria). These sequences were identical to haplotype S3 (LARGIADÈR *et al.*, 2000), which was already described for Plansee, as well as for several other localities in Northwestern Italy and Switzerland. Similarly, sequences of all the investigated specimens from the three Carinthian populations appeared to be identical. However, the haplotype found in these populations was different from haplotype S3 and did not match any of the GenBank sequences included in the analysis. The most closely related sequence was that from Las Lilas, France (accession number: AF237605), which differed by two substitutions (0.47%).

The final alignment comprised 19 sequences with a total of 431 characters. Altogether, 89 variable sites were found of which 30 characters were parsimony informative. MP yielded four most parsimonious trees with a length of 111 evolutionary steps (CI = 0.883, RI = 0.902). The topology of the strict consensus tree (not shown) closely resembled the NJ tree. Both methods corroborated the two major clades A and B, which were defined as *A. italicus* and *A. pallipes* by GRANDJEAN *et al.* (2002a, 2002b). Within clade A (*A. italicus*) the monophyly of sub-clades A1 (*A. italicus italicus*) and A3 (*A. italicus carsicus*) was supported by both MP and NJ, while sequences of sub-clade A2 (*A. italicus carinthiacus*) did not merge into a single cluster. This slight inconsistency could be due to the inclusion of an additional taxon or due to the analysis of a smaller number of characters (HILLIS, 1998; POE, 1998). Nevertheless, all populations from South Tyrol could be assigned to the *A. italicus carinthiacus* group, since all surveyed specimens carried haplotype S3 (LARGIADÈR *et al.*, 2000), which is already known from Plansee (GRANDJEAN *et al.*, 2002a, 2002b; LARGIADÈR *et al.*, 2000). In contrast, populations from Carinthia were shown to belong to a different clade – *A. italicus carsicus* –, which was found to be spread in France, Italy and Slovenia.

DISCUSSION

In the present study the actual phylogeny of the *A. pallipes* species complex was extended to several populations from the Alpine regions South Tyrol and Carinthia. The analysis of mtDNA sequences showed that all the investigated populations in South Tyrol carry the same haplotype (S3), which was shown to be distributed in Plansee (Austria) and at several localities in Switzerland and Northwestern Italy (LARGIADÈR *et al.*, 2000). Since this haplotype seems to have its natural distribution range in the Southern Alps, the only plausible explanation for finding it in Plansee in the Danube river basin, is human mediated colonization. On the one hand this assumption is reinforced by testimonies from elder residents (FÜREDER and MACHINO, 1995) and on the other by analyses of highly variable molecular markers, which clearly indicate a bottleneck event (BARIC *et al.*, 2005).



While the taxonomic status of the Plansee population of *A. pallipes* was determined in several previous works (GRANDJEAN *et al.*, 2000, 2002a, 2002b; LARGIADÈR *et al.*, 2000), this is the first study addressing the genetic composition of populations from

◀ **Figure 2**

Neighbor joining tree of the *A. pallipes* species complex based on partial mtDNA 16S rRNA sequences. *A. torrentium* was used as outgroup. Bootstrap values for the major nodes obtained through maximum parsimony and neighbor joining (1,000 replicates each) are given in boxes (MP above branches, NJ below branches). Sequences gained from GenBank are indicated by black letters, while sequences from newly analysed populations are given in bold letters. Bars denote the major clades and sub-clades of the *A. pallipes* species complex according to GRANDJEAN *et al.* (2002). The grey circle indicates haplotype S3 described by LARGIADÈR *et al.* (2000).

Figure 2

Arbre phylogénétique du complexe d'espèces *A. pallipes* construit par la méthode du neighbor-joining (NJ) basé sur les séquences partielles d'ADNmt 16s. *A. torrentium* a été utilisé comme extra groupe. Les valeurs de bootstrap pour les nœuds majeurs, obtenus par les méthodes du maximum de parcimonie (MP) et par neighbor-joining (1 000 répétitions chacune) sont données dans des cases spécifiques (MP sur les branches, NJ sous les branches). Les séquences issues de Genbank sont indiquées en lettres noires, tandis que les séquences provenant des populations nouvellement analysées sont données en gras. Les barres dénotent les clades principaux et les sous-clades du complexe d'espèces *A. pallipes* selon GRANDJEAN *et al.* (2002). Le cercle gris indique l'haplotype S3 décrit par LARGIADÈR *et al.* (2000).

Carinthia by using mtDNA sequences. Our data clearly demonstrate that the two unique occurrences of *A. pallipes* in the Danube river system are not related to each other and thus confirm the findings based on morphology (ALBRECHT, 1981; MACHINO, 1997; MACHINO and FÜREDER, 1996). Furthermore, our study also shows that there is no connection among white-clawed crayfish populations from South Tyrol and Carinthia. Although these populations belong to separate river basins, the Drave River (a major tributary of the Danube River) and the Rienz River (part of the Adige river system, which flows into the Adriatic Sea) originate in strong vicinity in the Carnic Alps. The divide of the two river basins is formed by the Toblacher Feld, with a height of 1,200 m above sea level. Keeping in mind that in some European regions the altitudinal limit of distribution of *A. pallipes* reaches up to 1,500 m above sea level (FÜREDER and MACHINO, 1999), one could have speculated that the “enigmatic” occurrences of white-clawed crayfish in Carinthia might be attributed to migration events from South Tyrol. Such presumptions can now be discarded by DNA sequence data, which place the Carinthian *A. pallipes* into a completely different phylogenetic lineage - *A. italicus carsicus*.

It is noteworthy that one single haplotype was found in all the surveyed specimens, which were collected from three geographically distinct localities within the Drave drainage – the Gitsch Valley, the Gail Valley and the Drave Valley (MACHINO, 1997; PETUTSCHNIG, 1998). This unique haplotype was discovered for the first time in the present study. Although it was possible to assess its phylogenetic relationships within the *A. pallipes* species complex, where the Carinthian haplotype clustered within the *A. italicus carsicus* clade together with haplotypes from France, Italy and Slovenia, mtDNA sequence data do not allow us to make any implications about the exact origin of *A. pallipes* in Carinthia. The answer to the question, whether these populations have naturally colonized this area and from where they originate, will have to be assessed in future studies by using more variable markers and investigating adjacent areas. Such markers will additionally provide valuable information about the genetic variability and structure of white-clawed crayfish populations in Carinthia, which are basic requirements for conservation issues. This was shown for South Tyrolean populations revealing a single mitochondrial haplotype of the

A. italicus carinthiacus lineage. However, the analysis of microsatellite DNA loci displayed high degrees of population differentiation on a small geographical scale showing the necessity to treat these populations as separate genetic units for conservation and management (BARIC *et al.*, 2005).

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