

COMPILATION OF MOLECULAR DATA FOR THE PHYLOGENY OF THE GENUS *AUSTROPOTAMOBIUS*: ONE SPECIES OR SEVERAL?

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ABSTRACT

The phylogenetic relationships of the genus *Austropotamobius* were reviewed by the compilation of two recent genetic studies based on the partial nucleotide sequence of the mitochondrial RNA 16S gene. The results showed a well-resolved phylogeny revealing four distinct clades: A1, A2, A3 and B, supported by high bootstrap values. The clades A (including A1, A2, A3) and B are separated by a high genetic divergence (5%). In accordance with morphological and nuclear data, two species could be defined: *A. italicus* and *A. pallipes*, respectively. The average of genetic divergence within the major group A and B was $2.1\% \pm 1.2$ and $0.9\% \pm 0.6$ respectively. Three clades A1, A2 and A3 correspond mainly to crayfish sampled from Austria-Switzerland, South of Balkans and Italy-Spain respectively. On the basis of morphological, genetic and distribution data, a new systematic - based on two species *A. pallipes* and *A. italicus* with 3 subspecies *A. i. carinthiacus*, *A. i. carsicus* and *A. i. italicus* - could be proposed for the white-clawed crayfish species complex. We rejected the specific status of *A. berndhauseri* and *A. i. lusitanicus* given to the Swiss and Spanish crayfish respectively.

Key-words : *Austropotamobius*, species complex, phylogeny, 16S RNA.

COMPILATION DE DONNÉES MOLÉCULAIRES POUR RÉSOLVRE LA PHYLOGÉNIE DU GENRE *AUSTROPOTAMOBIUS* : UNE OU PLUSIEURS ESPÈCES ?

RÉSUMÉ

Les relations phylogénétiques du genre *Austropotamobius* ont été revues en compilant deux récentes études génétiques basées sur la séquence nucléotidique partielle du gène mitochondrial codant pour la grande sous-unité de l'ARN ribosomal 16S. Les résultats ont montré une phylogénie robuste révélant quatre groupes : A1, A2, A3 et B, supportés par de fortes valeurs de bootstrap. Les groupes A et B sont séparés par une forte distance génétique (5 %). En accord avec les données morphologiques et nucléaires, deux espèces peuvent être définies : *A. pallipes* et *A. italicus*. La moyenne de divergence génétique à l'intérieur des groupes A et B est respectivement de $2,1\% \pm 1,2$ et $0,9\% \pm 0,6$. Les trois groupes A1, A2 et A3 correspondent principalement aux écrevisses échantillonnées en Autriche-Suisse, au Sud des Balkans et en Italie-Espagne

respectivement. Sur la base des données morphologiques, génétiques et géographiques, une nouvelle systématique - basée sur l'existence de deux espèces *A. pallipes* et *A. italicus* et trois sous-espèces *A. i. carinthiacus*, *A. i. carsicus* et *A. i. italicus* - peut être proposée chez le complexe d'espèces de l'écrevisse à pattes blanches. Nous rejetons les statuts taxonomiques spécifiques, *A. berndhauseri*, et *A. i. lusitanicus* donnés aux écrevisses suisses et espagnoles.

Mots-clés : *Austropotamobius*, complexe d'espèces, phylogénie, ARN 16S.

INTRODUCTION

Although all authors agree on specific status for the stone crayfish *A. torrentium* (BOTT, 1950; KARAMAN, 1963; ALBRECHT, 1982, 1983), the systematics of the white-clawed crayfish species complex remains controversial. Thus the systematics of the genus *Austropotamobius* is a matter of continuing debate, especially because morphological data provide ambiguous phylogenetic evidence. Several distinct classifications have been proposed by different systematists: some proposing one species and multiple subspecies or varieties while others recommend numerous species. The most significant taxonomic studies, based mainly on morphological criteria, are compiled in Table I:

(1) BOTT (1950, 1972) recognized two species: *A. berndhauseri* restricted to Southern Switzerland, more precisely in canton Tessin and southern valleys of the canton of Graubünden, and *A. pallipes* in western Europe (Table I). For *A. pallipes*, he described three sub-species: *A. p. pallipes* in Great Britain, Ireland, France, Switzerland and Corsica; *italicus* in Italy, Dalmatia and Switzerland and *lusitanicus* in the Iberian Peninsula. According to this author, *italicus* and *lusitanicus* differ from *pallipes* by the shape of the rostrum and its basal spines, while *italicus* and *lusitanicus* are separated by the presence of hairs on the upper border of the endopod of the second male gonopod in *lusitanicus*. However, such characters are of limited value for identifying specimens because they differ considerably from one population to another and the differences between sub-species are small. FROGLIA (1978) reported specimens in northern Piemonte and Liguria that had characters intermediate between *pallipes* and *italicus*. ALMACA (1987) and GRANDJEAN *et al.* (1998) found that most male *lusitanicus* specimens had no hairs on the second gonopod. LÖRSTCHER *et al.* (1997) reported specimen having intermediate morphological characters between *A. p. pallipes* and *A. berndhauseri*.

(2) KARAMAN (1963) named two species, *A. pallipes* and *A. italicus*, subdividing the latter into three subspecies *A. i. italicus*, *A. i. lusitanicus* and *A. i. carsicus*, with the last one distributed in the former Yugoslavia.

(3) ALBRECHT (1982) reported one species, *A. pallipes*, with several varieties in France, Spain, and Italy.

(4) STAROBOGATOV (1995) recognized 4 species with six sub-species (see Table I).

Where morphological data are inconclusive, genetic characters may provide accurate and unambiguous indicators of taxonomic divergence (WILSON *et al.*, 1985). The analysis of genetic structure of populations has recently led to the development of a new species concept that CRACRAFT (1983) called the Phylogenetic Species Concept (PSC). Based on the study of the genealogical relationship between taxa, the status of a taxon is related to its genetic uniqueness and whether or not it is monophyletic. However, the threshold for any taxonomic level is somewhat subjective. For this reason, AVISE and BALL (1990) recommend that morphological, biological and phylogenetic data be considered together to make a clear decision on the specific status of a group. At the intraspecific level, this method could be used for the definition of subspecies, when associated with the spatial distribution of lineages (AVISE *et al.*, 1987).

Table I

List of references to taxonomic studies of the *Austropotamobius pallipes* complex species.

Tableau I

Liste de références des études taxonomiques du complexe d'espèces *Austropotamobius pallipes*.

Authors	Genus	Species	Subspecies	Distribution	Data
BOTT (1950, 1972)	<i>Austropotamobius</i>	<i>pallipes</i>	<i>pallipes</i> <i>italicus</i> <i>lusitanicus</i> <i>berndhauseri</i>	France-British Isles Italy, Balkans Spain Switzerland	Morphology
KARAMAN (1963)	<i>Austropotamobius</i>	<i>pallipes</i> <i>italicus</i>	<i>italicus</i> <i>lusitanicus</i> <i>carsicus</i>	France-British Isles Italy Spain Balkans	Morphology
ALBRECHT (1982)	<i>Austropotamobius</i>	<i>pallipes</i>	<i>pallipes</i> <i>lombardicus</i> <i>carinthiacus</i> <i>trentinicus</i>	France Northern Italy Austria trentin	Morphology
BRODSKY (1983)	<i>Austropotamobius</i>	<i>pallipes</i> <i>italicus</i>	<i>pallipes</i> <i>bispinosus</i> <i>italicus</i> <i>lusitanicus</i> <i>carsicus</i>	France-British Isles Italy Italy Spain Balkans	Morphology Biogeography
PRETZMANN (1987)	<i>Austropotamobius</i>	<i>pallipes</i> <i>pallipes</i>	<i>pallipes</i> <i>fulcisanus</i> = <i>italicus</i>	France-British Isles-Spain-portugal-Switzerland North of Italy, Apennins, Dalamatia	Morphology Biogeography
STAROBORATOV (1995)	<i>Atlantoastacus</i>	<i>pallipes</i> <i>fulsinacus</i> <i>orientalis</i> <i>lusitanicus</i>	<i>pallipes</i> <i>rhodanicus</i> <i>fulsinacus</i> = <i>A. berndhauseri</i> + <i>A. p. lombardicus</i> + <i>A. p. trentinicus</i> <i>italicus</i> <i>orientalis</i> = <i>carsicus</i> <i>carinthiacus</i>	France-British Isles Switzerland-Trentin Italy Austria Balkans Spain	Morphology
GRANDJEAN <i>et al.</i> (1998)	<i>Austropotamobius</i>	<i>pallipes</i>	<i>pallipes</i> <i>italicus</i> <i>lusitanicus</i>	France-British Isles Balkans Spain	mt DNA by RFLP Morphology
GRANDJEAN <i>et al.</i> (2000)	<i>Austropotamobius</i>	<i>pallipes</i> <i>italicus</i> <i>berndhauseri</i> ?	<i>pallipes</i> <i>italicus</i> <i>carsicus</i> <i>carinthiacus</i>	France-England-Ireland Italy-Spain Balkans Switzerland-Austria Not sampled	mt 16S rDNA

In Astacidae and particularly in the white-clawed crayfish species complex, most recent molecular studies (Table II) have focused on the genetic structure of populations with no taxonomic inference (SANTUCCI *et al.*, 1997; LÖRSTCHER *et al.*, 1997; SOUTY-GROSSET *et al.*, 1997; GRANDJEAN *et al.*, 1997a, b, 2001; GRANDJEAN and SOUTY-GROSSET, 2000a, b; GOUIN *et al.*, 2001). ALBRECHT (1982) used allozyme electrophoresis to evaluate taxonomic issues within Astacidae, but the absence of polymorphism detected by this technique limited the informativeness of this study. Recently, SANTUCCI *et al.* (1997) and LÖRSTCHER *et al.* (1997) have revealed the efficiency of allozymes to study the genetic structure of Italian and Swiss populations respectively. Mitochondrial DNA (mtDNA) is a widely used marker for studying population differentiation because of its maternal, non-recombining mode of inheritance and rapid rate of evolution. Phylogeographic studies have shown that most species have hierarchical and sometimes deep genetic structures (AVISE, 1994) and specially in freshwater vertebrates (TSIGENOPOULOS and BERREBI, 2000). Recently, GRANDJEAN *et al.* (1998) reported the existence of three genetic entities among four European populations based on the analysis of total mitochondrial DNA by restriction fragment length polymorphism (RFLP) corresponding to the three subspecies described by BOTT (1950). But their results are of limited taxonomic values due to the restricted sampling area including only few samples from France, England, Spain and Slovenia.

More recently, based on the analysis of mitochondrial gene for the 16S RNA, an opinion has been expressed that the traditional morphology-based taxonomy is inconsistent with the white-clawed crayfish molecular phylogeny (GRANDJEAN *et al.*, 2000). They recommended that a taxonomic classification based on molecular phylogeny should be accepted. However, their study did not include a sample from Switzerland where *A. berndhauseri* (BOTT, 1972) or *A. fulcisanus* (STAROBOGATOV, 1995) occurs. However, LARGIARDÈR *et al.* (2000) published a similar study on Swiss populations. These two works gave the opportunity to re-examine the taxonomy of *A. pallipes* species complex over its range by the compilation of the whole of 16S RNA sequences. The phylogeny obtained is discussed with the light of recent nuclear genetic and morphological studies.

Table II
List of references on genetic studies conducted on the *Austropotamobius pallipes* species complex.

Tableau II
Liste de références des études génétiques sur le complexe d'espèces *Austropotamobius pallipes*.

Authors		Sampling location	Population Number	Markers
GRANDJEAN <i>et al.</i>	1997a	England	4	MtDNA RFLP
	1997b	Spain-Slovenia-France-England	5	
	1998	Spain-Slovenia-France-England	4	
	2001	Spain	14	
GRANDJEAN and SOUTY-GROSSET	2000a	France	10	
	2000b	France	25	
SANTUCCI <i>et al.</i>	1997	Italy	31	Allozyme
LÖRSTCHER <i>et al.</i>	1997	Swiss-Italy	19	
LARGIARDÈR <i>et al.</i>	2000	Swiss-Italy	25	
SOUTY-GROSSET <i>et al.</i>	1999	France	9	RAPDs
GOUIN <i>et al.</i>	2001	France-British Isles	15	

MATERIAL AND METHODS

Sampling - We examined 29 partial 16S rRNA sequences obtained from two studies performed by GRANDJEAN *et al.* (2000) and LARGIADÈR *et al.* (2000). The sequences have been deposited in GenBank under accession numbers AF237590-AF237610 (without AF237598: this sequence is very short compared to the others, with few unresolved sites) and AJ242700-AJ242711, in GRANDJEAN *et al.* (2000) and LARGIADÈR *et al.* (2000), respectively. The sequence (AF237599) corresponding to an individual of *A. torrentium* has been chosen as an outgroup. The sampling covers the entire range of white-clawed crayfish with animals coming from Austria, Great Britain, France, Ireland, Italy, Slovenia, Spain and Switzerland.

Alignment and phylogenetic inferences - Partial 16S rRNA sequences from *Austropotamobius pallipes* were manually aligned with the sequence of *Austropotamobius torrentium* (AF237599) as an outgroup. The phylogenetic analysis was performed on the resulting data matrix of 18 sequences (11 sequences were not included in this analysis due to redundancy of samples from the two previous studies) and 467 sites. Gaps were treated as missing data.

In phylogenetic estimation, it is necessary to justify a model of evolution used to model character-state changes (HUELSENBECK and CRANDALL, 1997). We used the program ModelTest (POSADA and CRANDALL, 1998) to test alternative models of evolution for our data using likelihood ratio tests. The program tests a number of null hypotheses, including: (1) equal base frequencies, (2) equal transition/transversion rates, (3) equal rates within transition and transversion classes, (4) equal rates of substitution and (5) no invariable sites. We tested all these hypotheses in order to optimize a model of evolution. Phylogenetic trees were inferred using PAUP 4.0b8a software (SWOFFORD, 1998). Tree reconstructions were performed using maximum likelihood and distance methods assuming the substitution model selected by ModelTest and rate heterogeneity across sites. The likelihood search was performed using random sequence addition and setting parameters to values indicated in the model optimisation. The search was heuristic based on branch swapping by tree bisection-reconnection (TBR). Computation of distances was done using the neighbour joining algorithm (SAITOU and NEI, 1987). Bootstrap analysis was performed with 1 000 pseudoreplicates (FELSENSTEIN, 1985).

RESULTS

Within the data matrix of 18 sequences, we rejected the null hypothesis of equal base frequencies. There appears to be an AT bias in these data (base frequencies: A = 0.3383, C = 0.1161, G = 0.2103 and T = 0.3353). The transition/transversion ratio was 2.3463. The model of substitution selected by hierarchical likelihood ratio tests was therefore the HKY85 model (HASEGAWA *et al.*, 1985) with a gamma distribution shape parameter estimated to 0.672. Figure 1 shows the tree inferred from ML analysis (ln likelihood value of -1162.69). Irrespective of the two methods employed (distance or ML), all phylogenetic inferences exhibited identical topology.

The phylogenetic analyses support the separation of the four clades (labelled A1 including mainly individuals from Italian, Spanish and French specimens, A2 including mainly individuals from Austrian and Swiss ones and A3 including mainly individuals from Slovenian and B including individuals from England, France, Corsica and Ireland). All clades are supported by high bootstrap values (Figure 1).

The average of genetic divergence within the major group A (including A1, A2 and A3) and clade B were $2.1\% \pm 1.2$ and $0.9\% \pm 0.6$, respectively (Table III). The average between the major group A and the clade B was $5.0 \pm 1\%$. Range of different taxa are illustrated in Figure 2.

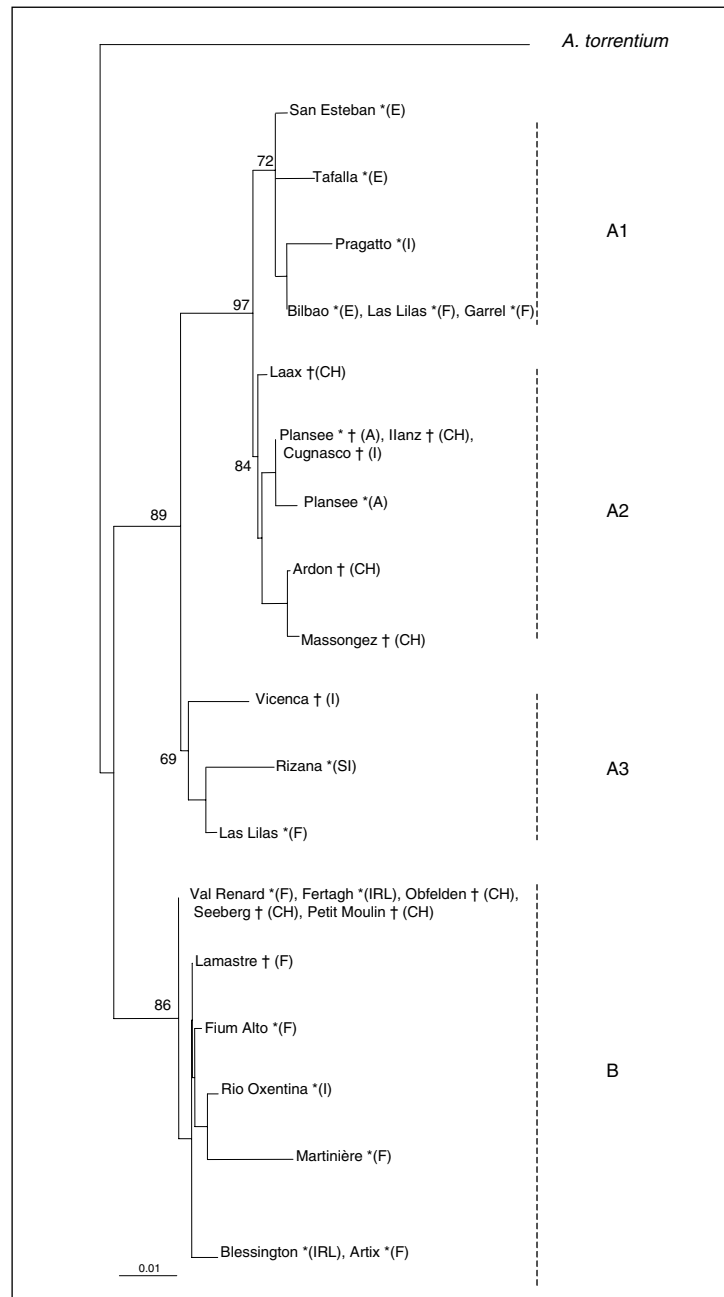


Figure 1

Maximum likelihood tree derived from analysis based on mt 16S rDNA characters using the HKY85 model (HASEGAWA *et al.*, 1985) from PAUP (4.0b8a version: SWOFFORD, 1998) rooted using *A. torrentium*. Numbers at nodes indicate bootstrap support $\geq 60\%$ (1 000 replicates). Populations labelled † or * are issued from LARGIADÈR *et al.* (2000) and GRANDJEAN *et al.* (2000) respectively. The clades labelled A1, A2 and A3 correspond to the subspecies of *A. italicus*: *A. i. italicus*, *A. i. carinthiacus* and *A. i. carsicus*, respectively and the clade B to *A. pallipes*.

Figure 1

Arbre obtenu par la méthode du maximum de vraisemblance à partir des séquences mitochondriales de l'ADNr 16S sous le modèle HKY 85 (HASEGAWA *et al.*, 1985) dans PAUP 4.0b8a version : SWOFFORD, 1998) raciné par *A. torrentium*. Les valeurs de bootstrap ($\geq 60\%$) sont indiquées au niveau des nœuds (1 000 réplicats). Les populations marquées † et * sont issues de LARGIADÈR *et al.* (2000) et GRANDJEAN *et al.* (2000) respectivement. Les groupes A1, A2 et A3 correspondent aux sous-espèces d'*A. italicus* : *A. i. italicus*, *A. i. carinthiacus* et *A. i. carsicus*, et le groupe B à *A. pallipes*.

Table III

Pairwise genetic divergence mean within and among species or subspecies.

Tableau III

Divergence génétique moyenne inter et intra taxons.

	Clade A1 (<i>A. i. italicus</i>)	Clade A2 (<i>A. i. carinthiacus</i>)	Clade A3 (<i>A. i. carsicus</i>)	Clade B (<i>A. pallipes</i>)
Clade A1	0.009 ± 0.005	0.014 ± 0.005	0.036 ± 0.007	0.054 ± 0.009
Clade A2		0.007 ± 0.004	0.030 ± 0.006	0.051 ± 0.008
Clade A3			0.019 ± 0.005	0.043 ± 0.01
Clade B				0.009 ± 0.006

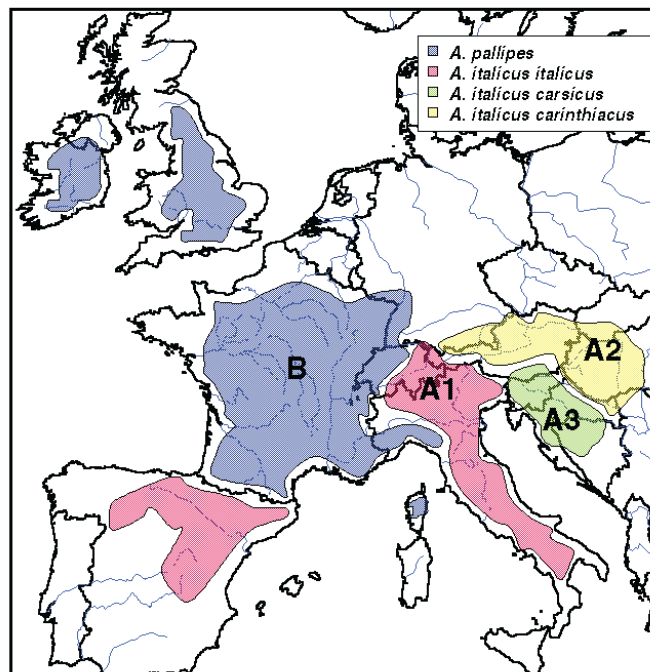


Figure 2

Range of taxa described in the white-crayfish species complex.

Figure 2

Aire de distribution des taxons décrits au sein du complexe d'espèces d'écrevisse à pattes blanches.

DISCUSSION

The results of this study reveal a well-resolved phylogeny. Maximum likelihood analysis identified two major reciprocal monophyletic groups of mtDNA sequence in the *A. pallipes* species complex (Clades A and B). Haplotypes from clade A are found in crayfish from France, Ireland and North of Italy and those of type B have been characterized from crayfish distributed in Switzerland, Austria, Italy, Balkans and Spain. These groups are strongly supported by the highest bootstrap percentages. Furthermore, they show high levels of genetic divergence on average 5% with a range of 4.3 to 5.4%. These two clades correspond perfectly with those previously reported Type A and B

described by GRANDJEAN *et al.* (2000). This is supported by other data sets such as nuclear markers and morphology. Indeed, in recent studies on allozymes, SANTUCCI *et al.* (1997), LÖRSTCHER *et al.* (1997) and LARGIARDÈR *et al.* (2000) also revealed a strong genetic structure among populations located in France and England and those sampled in Italy, Slovenia and Switzerland. SANTUCCI *et al.* (1997) reported an average Nei's genetic distance between these two clades of 0.30 (range 0.26 - 0.40). Moreover, they revealed a lack of F1 hybrids in sympatric areas which demonstrates their reproductive isolation in the field. Morphological results also are concordant with genetic data from mitochondrial and nuclear markers. LAURENT and SUSCILLON (1962) and GRANDJEAN *et al.* (1998) reported a A/R ratio (apex to rostrum length ratio) of approximately 0.22 for populations sampled in France and England, while populations from Balkans, Italy and Spain had ratios greater than 0.29. Similarly, the number of spines behind the cervical groove also allowed the discrimination of two geographically separated clades (LAURENT and SUSCILLON, 1962; ALBRECHT, 1982; GRANDJEAN *et al.*, 1998). Individuals from clade A had a number of spines significantly higher (mean 2.8) than those from clade B (mean 1). All these results showed that these two clades appear to constitute separate lineages with a long history of independent evolution. According to AVISE and BALL (1990), concordant divergence among clades for two or more unlinked markers could be candidates for species that we recognized as being *A. italicus* and *A. pallipes* for the clades A and B respectively.

Within the major group A, three well resolved clusters A1, A2 and A3 are defined with high bootstrap values ($\geq 69\%$). These clades correspond to those found by GRANDJEAN *et al.* (2000). No additional branch occurs, waiting for the addition of a new species (*A. berndhauseri* included in the study of LARGIARDÈR *et al.* (2000)) in the phylogenetic analysis. The *A. berndhauseri* specimens are clustered with Austrian ones into clade A2. This result is in accordance with the geographical closeness of these populations. In their studies, LARGIARDÈR *et al.* (2000) showed that populations of crayfish from Clade 2 are widespread in Southern Switzerland. Moreover A2 is genetically closer related to A1 including haplotypes from Italian-Spanish crayfishes than the clade A3 harbouring haplotypes from Slovenian ones. Thus this phylogenetic analysis does not confirm the specific status of *A. berndhauseri* given by BOTT (1972) to Swiss crayfish. The Austrian populations of crayfish have been morphologically studied by MACHINO (1997). This author confirmed the variety *carinthiacus* described by ALBRECHT (1982) from Austrian crayfish. According to these data, clade 2 could correspond to *A. italicus carinthiacus*. This phylogeny also lies with the placement of *A. italicus lusitanicus* specimen described as being endemic from Spain. Haplotypes from Spanish specimens are similar with those found in Italian samples. Recent population studies based on genetics and/or morphology are congruent with our data. GRANDJEAN *et al.* (2000) and SANTUCCI *et al.* (1997) revealed an absence of genetic differentiation between Spanish and Italian specimens from both mtDNA 16S RNA and allozyme markers. In a recent study to survey the extent and the pattern of genetic variation across the Spanish range of this species, GRANDJEAN *et al.* (2001) found one haplotype from the 154 animals sampled from 14 Spanish populations. This haplotype was also recorded in Italian populations, which populations showed a high intrapopulation variability. In agreement with the close genetic relationship based on both genetic markers, the authors assumed a translocation of crayfish from Italy to Spain and avoided the traditionally accepted subspecific status recognized for Spanish specimens. These results were concordant with morphological data (ALMACA, 1987; GRANDJEAN *et al.*, 1998). This example clarifies the taxonomic confusion existing within this species-complex before the application of genetic markers.

No intra-subdivisions were supported within clade B. *A. pallipes* seems to be the unique taxon. In the light of molecular studies, including samples covering the entire ranges of the *A. pallipes* species complex, a new systematics can be proposed for the white-clawed crayfish species complex based on two species *A. pallipes* and *A. italicus* with 3 subspecies *A. i. carinthiacus*, *A. i. carsicus* and *A. i. italicus*.

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