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The first record of translocated white-clawed crayfish from the *Austropotamobius pallipes* complex in Sardinia (Italy)

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ABSTRACT

The white-clawed crayfish *Austropotamobius pallipes* complex is native to Europe, being present in 18 European countries, Italy included. However, the number and abundance of its populations are today restricted and it has been recently classified as endangered by IUCN (International Union for Conservation of Nature). Here, we report the first record of this freshwater crayfish in Sardinia Island (Italy). Using a fragment of the mitochondrial DNA 16S rRNA gene, we identified three haplotypes that correspond to the *A. italicus meridionalis* subclade. We provide information about the sampling area, population density and finally discuss hypotheses about the occurrence of this population in Sardinia, comparing it with other Mediterranean populations. Our results improve the existing knowledge about the phylogeography of the taxon across Italy, confirming its complex pattern of distribution. In addition to the non-native status of the Sardinian *A. i. meridionalis* crayfish, we showed that the most proximal Mediterranean population of white-clawed crayfish existing in Corsica belongs to *A. pallipes* from Southern France.

Key words: *Austropotamobius pallipes* species complex, DNA barcoding, endangered species, Sardinia, Corsica.

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INTRODUCTION

The white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) has a wide geographic range in Europe, from Scotland in the North to Spain in the South and from the European Atlantic front in the West to the Montenegro in the East (Souty-Grosset *et al.*, 2006; Holdich *et al.*, 2009). Populations of this species have been recorded in 18 European countries, but those from Austria, England, Ireland, Portugal, Eastern Spain and Wales, as well as from Corsica, Liechtenstein and Scotland, appear to have originated from human introductions (Grandjean *et al.*, 2001; Gouin *et al.*, 2003; Souty-Grosset *et al.*, 2006; Holdich *et al.*, 2009). Since the 1860s, the number of *A. pallipes* populations has been sharply decreased due to the combined effects of several drivers of environmental change; *i.e.*, habitat loss and degradation, water pollution, overfishing or competition from non-indigenous crayfish species. The European native crayfish *A. pallipes* is also facing the spread of the crayfish plague, a lethal disease caused by the oomycete *Aphanomyces astaci* (Schikora, 1903) (Souty-Grosset *et al.*, 2006). As a result of its rarefaction, even leading to the extinction of the species in Portugal (Holdich *et al.*, 2009), *A. pallipes* is included in Annexes II and V of the European Union Habitats Directive

(92/43/EEC) as a species requiring special conservation measures. It has also been recently promoted by the International Union for Conservation of Nature (IUCN) from the status of *vulnerable* (Baillie *et al.*, 1996) to the status of *endangered* (Füreder *et al.*, 2010).

For a decade, many attempts have been made to conserve and restore *A. pallipes* populations (Souty-Grosset *et al.*, 2003, 2006; Souty-Grosset and Reynolds, 2009). However most of these actions suffered from the absence of a well-defined taxonomy (Fratini *et al.*, 2005; Manganello *et al.*, 2006). Indeed, the species shows a large genetic heterogeneity that might have originated from the climate changes occurring in Pleistocene and its confinement in glacial refugia (Santucci *et al.*, 1997; Pedraza-Lara *et al.*, 2010). Numerous taxonomic revisions have been done over the last five decades (Bott, 1950; Karaman, 1962; Starobogatov, 1995). As well as the taxonomical status of the *A. pallipes* species remains as unsolved and uncertain, some genetic studies based on sequences of the 16S rRNA and CO1 mitochondrial genes (Grandjean *et al.*, 2000, 2002; Matallanas *et al.*, 2011), partially supported by morphological (Bott, 1950; Karaman, 1962; Brodski, 1983; Grandjean *et al.*, 1998; Bertocchi *et al.*, 2008a) and allozyme data (Santucci *et al.*, 1997), suggest the existence of two different lineages (Grandjean *et al.*,

2000; but see Chiesa *et al.*, 2011; Scalici and Bravi, 2012). The first is *A. pallipes*, distributed across most river basins from France, the Rhine and Rhône drainages of Switzerland, the North-western Italy, the South-western tip of Germany and the British Isles (Grandjean *et al.*, 2002; Zaccara *et al.*, 2004; Souty-Grosset *et al.*, 2006; Stefani *et al.*, 2011). The second species is *A. italicus* (Faxon, 1914), present in Italy, Dalmatia and the Iberian Peninsula (Grandjean *et al.*, 2000; Fratini *et al.*, 2005; Souty-Grosset *et al.*, 2006). The latter was further distinguished into four subspecies with a well-defined geographic distribution, *i.e.*, *A. i. italicus* (Faxon, 1914), mainly found in the Central Apennines in Italy, two localities in the South of France and in Spain, *A. i. carsicus* (Karaman, 1962), distributed across the Dinaric and Eastern Alps, *A. i. carinthiacus* (Albrecht, 1981), found in Central and North-Western Italy, Austria and Switzerland, and *A. i. meridionalis* occurring in Southern Italy and Slovenia (Fratini *et al.*, 2005). However, *A. italicus* has not yet been officially recognized as a separate species (Grandjean *et al.*, 2000), and the systematics of *A. pallipes* is still under debate (Trontelj *et al.*, 2005). Several authors have thus decided to adopt the cautious approach of retaining *A. pallipes* as a species complex (Souty-Grosset *et al.*, 2006; Reynolds and Souty-Grosset, 2012). The present study acknowledges the existence of a species complex comprising *A. pallipes* and four subclades of *A. italicus*.

The taxonomic status of the crayfish populations inhabiting the Italian islands is still unknown. In continental Italy, the species occupies three different climatic areas, with altitudes comprised between 90 and 1050 m asl, from coastal areas on the Mediterranean Sea to the Alps and the Apennines (Holdich *et al.*, 2009). Aquiloni *et al.* (2010) argued that its current distribution is the result of two distinct factors; the first is the competition with the river crab, *Potamon fluviatile* (Herbst, 1785) (Barbaresi and Gherardi, 1997; Gherardi and Cioni, 2004), and which may have started in the Pleistocene when the two species met after their migration from Eastern Europe (Pretzmann, 1987). The second factor is the frequent release by humans of crayfish into the wild. Populations of the *A. i. meridionalis* subclade, for example, occur in Tuscany, where instead *A. i. italicus* is to be expected (Fratini *et al.*, 2005). Indeed, voluntary introduction of crayfish associated with their use as a food delicacy is a common practice in Western Europe, as abundantly documented in the literature (Grandjean *et al.*, 1997, 2000, 2001, 2002; Largiadèr *et al.*, 2000; Gouin *et al.*, 2003; Machino *et al.*, 2004). Here, we report the first finding of the species on Italian islands, *i.e.*, Sardinia; this new population was then genetically compared with other Italian and European populations to find possible relationships by using the mt DNA 16S rRNA. Moreover, we added some new genetic data of the Corsican white-clawed crayfish as the most proximal population to

the Sardinian one, to elaborate the most parsimonious scenario explaining its origin in Sardinia.

METHODS

Study area

The sampling took place at the stream called Basile, part of the Coghinas hydrographic basin in Sardinia Island, Italy (about 50 km from Sassari; 40°23'21.1"N, 8°54'17.9"E; altitude: 575 m asl). Isolated from inhabited areas, it is classified as *salmonid water* by the local Fish and Wildlife Offices (T: 15°C; oxygen: 8.9 mg/L; conductivity: 190 µS/cm; pH: 7.87). The stream bottom is covered by cobble, stones and boulders that, together with the abundant tree roots, are known to provide shelter to crayfish (Naura and Robinson, 1998). The area is part of a Site of Community Interest (SCI) under the Habitats Directive (Catena del Marghine Goceano). Due to the altitude range determining a wide climatic and phytoclimatic diversification, 750-800 species, comprising 38 endemisms and many plant species of phytogeographic relevance, occur, along with nine natural habitat types of community interest (three of which of priority relevance) and 28 threatened species of vertebrates (three mammals, 15 birds, six reptiles and four amphibians). Freshwater animal species of conservation concern include *Emys orbicularis* (Linnaeus, 1758), *Salmo cettii* (Rafinesque 1810), and *Euproctus platycephalus* (Gravenhorst, 1829).

Population density

In June and August 2010, after a preliminary inspection of the site, crayfish that at first glance were assigned to the *A. pallipes* complex were collected by hand by three persons walking upstream for 90 min during the night. This in-depth survey was conducted by turning rocks and investigating among roots and detritus. Immediately upon capture, sex was noted and the cephalothorax length (CL), including rostrum, was measured using a Vernier caliper. The occurrence of scars, mutilations, and visible ectosymbionts (*e.g.*, Branchiobdellidans) was recorded. After measurements, crayfish were released at the collection site.

We computed the catch per unit effort (CPUE, the number of crayfish divided by the time spent for sampling; individuals min⁻¹; Demers and Reynolds, 2002; Scalici and Gibertini, 2005) and density (individuals m⁻²).

Genetic analysis

A piece of claw was taken from each collected individual, preserved and stored in 95° ethanol for this study and for future genetic analyses. This does not cause any damage to the animal because appendages regenerate after successive moults. The DNA was extracted following a standard

Phenol-Chloroform extraction protocol. The amplification of a fragment of the mitochondrial DNA 16S rRNA gene (412 bp) was performed by PCR using two primers: 16 S-ar (5' - CGCCTGTTTAACAAAGACAT - 3') and 16 S-br (5' - CGGTCTGAACTCAGATCACGT - 3') (Palumbi *et al.*, 1991).

The following PCR conditions were applied: 40 cycles for 1 min at 95°C for denaturation, 1 min at 50°C for annealing, 1 min at 72°C for extension, preceded by 5 min of initial denaturation at 93°C and followed by 5 min of final extension at 72°C. PCR products were visualized in 1.5% agarose gels (1% TBE) stained with SYBR-Safe (Lonza, Belgium). The DNA size marker used was the Smart ladder Eurogentec® (200 to 10,000 pb). Successful double-strand PCR products were purified with the Exo-SAP-IT buffer (USB, Cleveland, OH, USA) and then sequenced using the Big Dye v. 3.1 Terminator Kit on a capillary automated sequencer ABI PRISM 3130 (PE Applied Biosystem, Carlsbad, CA, USA). For most samples, the forward and reverse sequences were obtained. In addition to the Sardinian individuals, we sequenced some specimens from Corsica and France; all the haplotypes are available in GenBank under the accession numbers KP866104-KP866115. Sequences from Grandjean *et al.* (2000), Largiadèr *et al.* (2000), Zaccara *et al.* (2004), Fratini *et al.* (2005) and Pedraza-Lara *et al.* (2010) were added to the sample dataset to increase the accuracy of the analysis.

Phylogenetic inference

We restricted the location selection to Italy and Southern France. Sequences from *A. torrentium* from Switzerland were added as an outgroup to the overall dataset. First, we used a Bayesian inference implemented in BEAST 1.8.0 (Drummond *et al.*, 2012) for computing the species phylogeny and detecting the taxonomical affiliation of the Sardinian population. We selected the HKY (Hasegawa *et al.*, 1985) substitution model in MEGA 6 (Tamura *et al.*, 2013) with selection on the lowest Akaike Index Criterion (AIC). Three Metropolis-coupled Markov chains (MCMC) were run 10 million generations with trees sampled every 1,000 generations. We used TRACER 1.6 (<http://beast.bio.ed.ac.uk/Tracer>) to check that all Effective Sample Size (ESS) were largely exceeding the minimum value of 100 to ensure the meaningful estimation of parameters. The different runs were combined using LogCombiner 1.8.0. (<http://beast.bio.ed.ac.uk/logcombiner>). We discarded the first 10% trees in TreeAnnotator 1.8 (<http://beast.bio.ed.ac.uk/treeannotator>). Then, we visualized the trees topologies in FigTree 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>). Secondly we added to the posterior probabilities of the Bayesian inference, the bootstrap values from a Maximum Likelihood analysis (1000 repetitions, MEGA 6; Tamura *et al.*,

2013), to support the main branches of the species complex phylogeny.

Phylogeographic origin

F-statistic (Φ_{ST}) and its probability values (P-values) were computed in Arlequin 3.1 (Excoffier *et al.*, 2005), with 1000 permutations, for each geographic unit of the subspecies *A. i. meridionalis*; e.g., Po basin including several populations [Prato (5), Bormida (10), Vignole A. (10) and Visone (10)], Southern Italy [Crati (8), Sele (15) and Umbria (18)], and Sardinia (Fig. 1). We reported the same divergence indices between the Corsican population and the *A. pallipes* populations from France and Northern Italy (Po River).

RESULTS

Morphological description

We checked all the diagnostic characters for *A. pallipes* as reported by Holdich *et al.* (2006). One individual is shown in Fig. 2. Colour is brown to olive with pitted appearance; the maximum total length recorded was 7 cm. The carapace was smooth with a pitted appearance with a single pair of post-orbital ridge on it. A row of tubercles was found on the shoulder of the carapace behind the cervical groove. The ends of pleura from the second to the fourth abdominal somites were rounded without sub-terminal spines. The areola between branchiocardiac grooves was evident. The rostrum was broad-based, with smooth borders converging to a long sharp acumen; a simple median carina runs down its length; the dorsal surface was covered in mat of fine setae. The topside of the chelae was weakly granular and the underside was dirty-white colour. Two widely spaced tubercles were present on the inner side of the fixed finger with obvious incision between them; no prominent spur was found on the inner margin of the cheliped carpus.

Population description

We recorded 188 crayfish (95 males and 93 females) and the sex ratio did not significantly differ from 1:1. CPUE and density were of 2.08 ind.m⁻¹ and 2.03 ind.m⁻² respectively. Overall, 12% crayfishes were found without a cheliped and 17.7% had a regenerated one. No scars or ectosymbionts were found and no individual showed apparent symptoms of either thelohanian or aphanomycosis.

Phylogenetic inference

The data matrix of 223 mitochondrial DNA 16S rRNA gene sequences of *Austropotamobius* spp., consisted of eight samples from Sardinia, 19 from Corsica, nine from the Rhône (France) and six samples from the French Eastern Pyrénées, were supplemented by 181 sequences re-

trieved from GenBank (Tab. 1). The topology of the Bayesian phylogenetic tree (Fig. 3) allowed us to affiliate the Sardinian samples as a population of *A. i. meridionalis*. Moreover, we noticed that the added samples from Corsica belonged to the *A. pallipes* clade.

Phylogeographic origin

Within *A. i. meridionalis*, we found no genetic differentiation between Sardinian population and South Italian populations [Crati (8), Sele (15 and Umbria (18)] (Tab.1) nevertheless, the pairwise Φ_{st} values between the Sardinian population and the *A. i. meridionalis* from the Po River (Bormida [10], Vignole A. [10] and Visone [10], Tab. 1) showed a significant genetic differentiation: $\Phi_{st}=0.280$, $P<0.05$. We also noticed a slight differentiation between the Sardinian and the Southern Pyrenean sample but in a lower order of magnitude: $\Phi_{st}=0.109$, $P=0.06$. The pairwise Φ_{st} value between the Po River and Southern Italy was slightly the same between Sardinia and the Po River: $\Phi_{st}=0.306$, $P<0.05$.

The Corsican population displayed larger significant

divergence with the other population of *A. pallipes* of the present study (vs Rhône; $\Phi_{st}=0.482$, $P<0.001$ and vs the Po River; $\Phi_{st}=0.310$, $P<0.001$). However, both Tyrrhenian populations were highly significantly differentiated ($\Phi_{st}=0.951$, $P<0.001$).



Fig. 2. An individual of *Austropotamobius italicus meridionalis* from the study site in Sardinia.

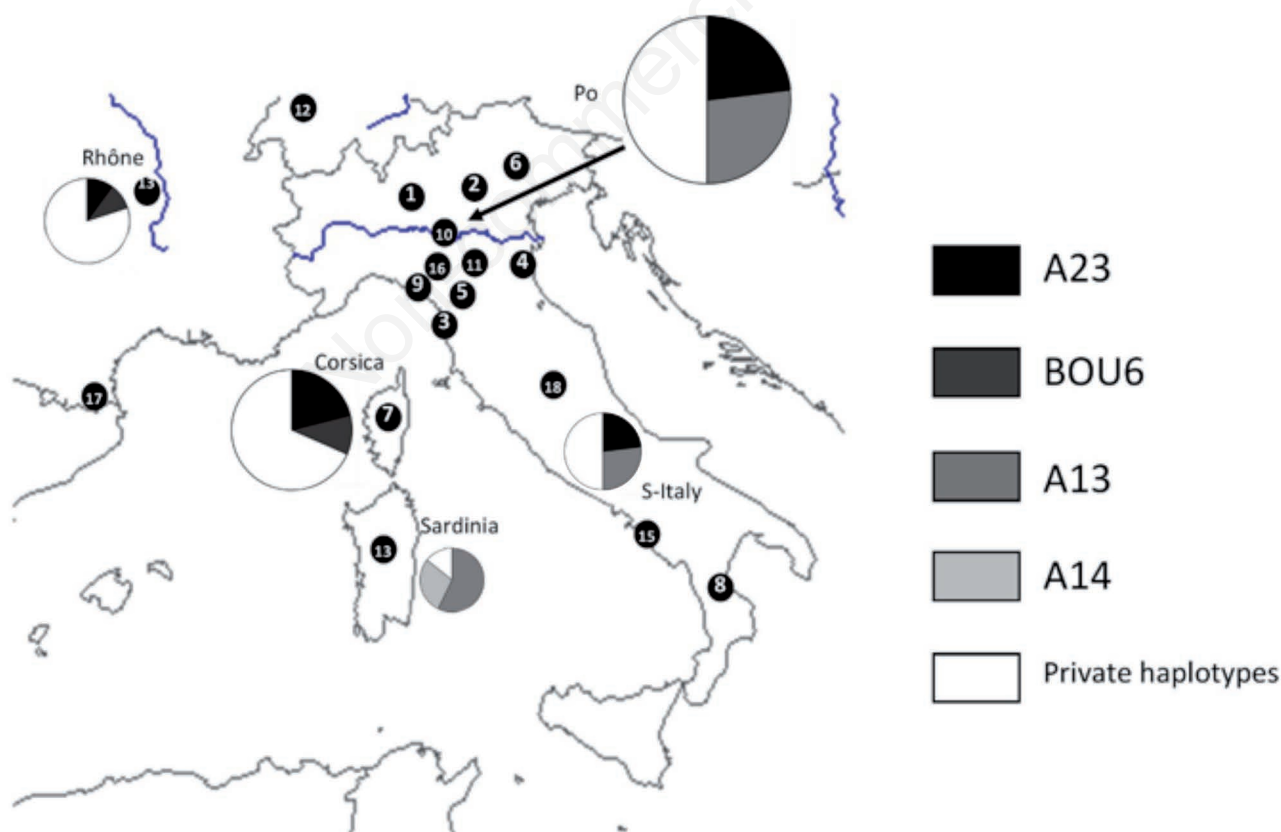


Fig. 1. Locations of the different populations of *Austropotamobius* sp. Haplotypes restricted to one sampling location (i.e., private haplotypes) are represented in white within pie charts while haplotypes shared between locations are shaded using a grey scale. The size of pie charts is proportional to the sampling size.

Tab. 1. Populations of *Austropotamobius pallipes* complex sampled in Sardinia (Italy) and at different neighboring locations included and analyzed in this study. For each population, basin, # reference number on Fig. 1, number of individuals (N), mt16S rRNA, haplotype composition are reported.

Basin	# Fig.1	Taxon	<i>A.i. carinthiacus</i>	<i>A.i. carsicus</i>	<i>A.i. italicus</i>	<i>A.i. meridionalis</i>	<i>A. pallipes</i>	<i>A. torrentium</i>
mtDNA haplotype			A1 A10 A2 A15 A16 A17 A18 B1 B2 V1 V2 Q2 A6 A7 A8 Ad F590 H I2 I3 I4 I5 LAS13 Ver A13 A14 A9 I6 LAS1 SARD4 A23 A24 BOU1 BOU17 BOU6 CAS1 COR2 COR4 ESA3 F596 LAS2 L553 N1 N2 POL1 RR2 SS2 SUI3					
Locality (Country)								
N								
Adda 1	1	Sondrio (IT)	1	1				
Adige 1	2	Monti Berici (IT)	4	1 3				
Arno 1	3	Arezzo (IT)	17	10	3 1 3			
Arno 1	3	Farfeta (IT)	2	1 1				
Arno 1	3	Resco Simontano (IT)	4	4				
Arno 1	3	Staggia (IT)	2	2				
Bidente-ronco 1	4	Lama (IT)	2	2				
Bisenzio 1	5	Prato (IT)	8		2 3 3	3		
Brenta 1	6	Lake Caldonazzo (IT)	1	1				
Corsica 2	7	Casabianca (FR)	3			1 1 1		
Corsica 2	7	Esao Ficaja (FR)	6			3 1	2	
Corsica 2	7	Fiume alto (FR)	4				2 1 1	1
Corsica 2	7	Polveroso (FR)	6					6
Crati 1	8	Coscile (IT)	2		2			
Magra 1	9	Collegnago (IT)	2 2					
Magra 1	9	Gottero (IT)	2 2					
Magra 1	9	Massa (IT)	2		2			
Po 1	10	Adda (IT)	7	4 3				
Po 1	10	Borbera-Lagoscuro (IT)	6 2 4					
Po 1	10	Bornida (IT)	3					
Po 1	10	Brescia (IT)	1	1				
Po 1	10	Clivio (IT)	4 3 1					
Po 1	10	Coppa (IT)	2 2					
Po 1	10	Gumpen (IT)	1 1					
Po 1	10	Lambro (IT)	18 10 2 3 1 1					
Po 1	10	Lazzuola (IT)	3 3					
Po 1	10	Lemme (IT)	9 5			2		2
Po 1	10	Malone (IT)	2					2
Po 1	10	Montenotte (IT)	1			1		
Po 1	10	Nemmo (IT)	1					
Po 1	10	Ora (IT)	2	2				

To be continued on next page

Table 1. Continued from previous page.

1, data from previous published studies (Fratini et al., 2005 ; Grandjean et al., 2000; Largiadèr et al., 2010; Pedriza-Lara et al., 2004). 2, data obtained from the present study.

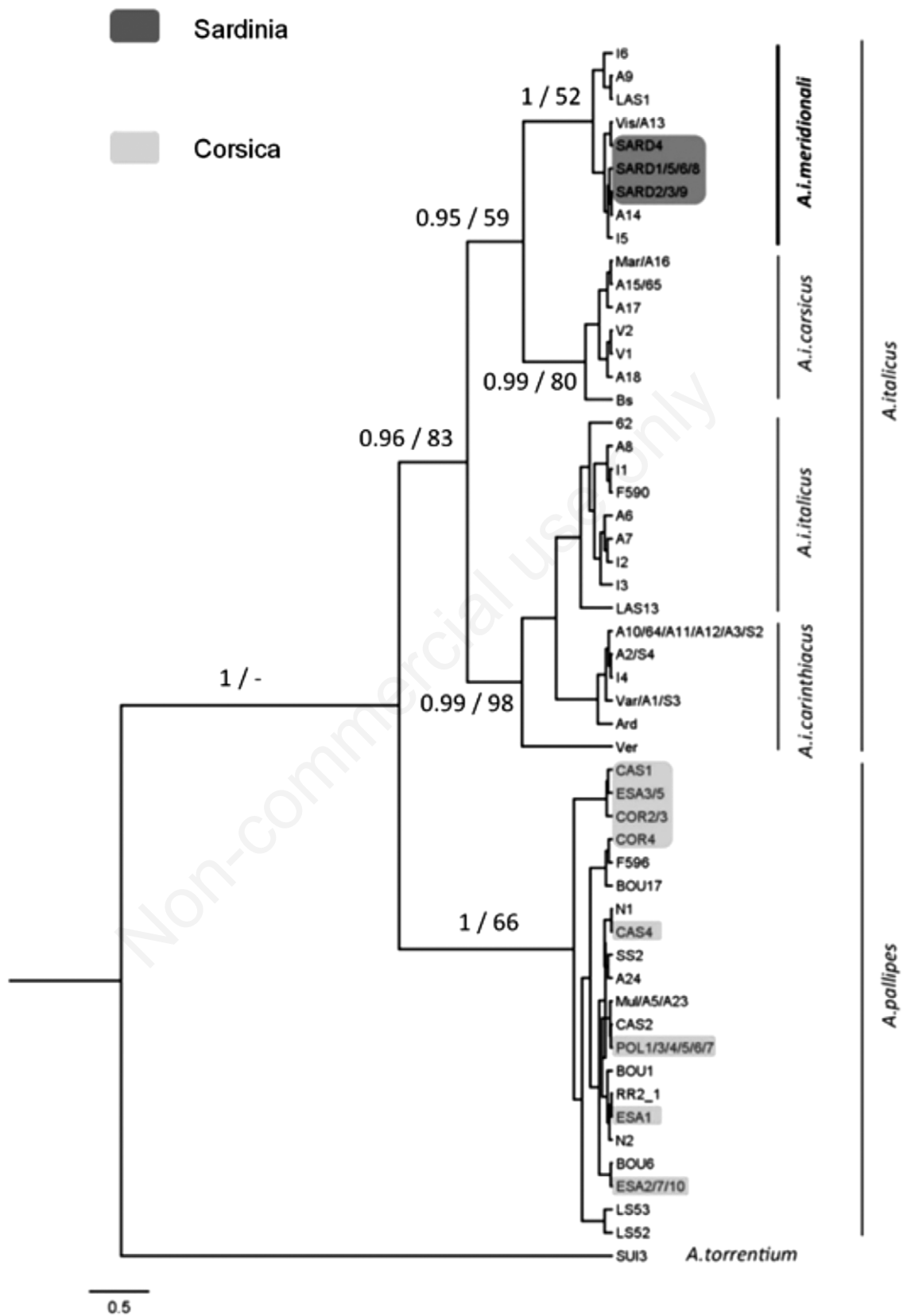


Fig. 3. Bayesian consensus tree based on mtDNA 16S rRNA haplotypes. Numbers above branches are the Bayesian posterior probabilities and the bootstrap values from Maximum Likelihood inference respectively.

DISCUSSION

Considering the decline reported for *A. pallipes* complex in other Italian regions and European countries (Füreder *et al.*, 2003; Lyons and Kelly-Quinn, 2003; Scalici and Gibertini, 2005), the study population seems to be healthy, similarly to some populations sampled in Tuscany, Central Italy (Brusconi *et al.*, 2008). It shows indeed high CPUE values (0.93 maximum in Scalici and Gibertini, 2005; 2.12 maximum in Brusconi *et al.*, 2008), a balanced sex-ratio, a relatively low number of injured individuals, and no scars, ectosymbionts or signs of diseases. Our phylogenetic analysis reveals that the Sardinian population of crayfishes under study belongs to the *A. i. meridionalis* subclade, which thus extends its distribution from France, Spain, Slovenia and continental Italy to the Sardinia Island.

If this Sardinian population might result from a natural genetic divergence, both the Pleistocene climate and the regional geomorphological history might have influenced the splitting of species. As shown in *Proseallus* species (Matthaeis *et al.*, 2001), the natural genetic divergence of the Sardinian population is the largest within its Mediterranean distribution. This is not the case in our dataset; the Sardinian population is genetically very similar to the Southern Italian populations of *A. i. meridionalis*. However, given that the separation of the Corsica-Sardinia microplate ranged between 50-25 million years before present (MYBP) and 16-12 MYBP (Alvarez, 1972), the genetic pool of any endemic population in Sardinia would have been much more differentiated from the other Italian and European populations. This hypothetically isolated relic population would have shown a unique genetic pool characterised by a much more stronger genetic divergence than we have found in this study. Consequently, a recent human-mediated translocation seems to be the most probable explanation of our findings because of the close similarity of the Sardinian genetically type with other Southern Italian populations of *A. i. meridionalis*; *i.e.*, we found low and non-significant F-statistics values with the populations from Sele, Crati and Umbria. Recent studies revealed that translocation events are numerous as discovered both in Maritim alps (Stefani *et al.*, 2011) and the record of a population of the southern lineage of *A. italicus*; *i.e.*, a non-native taxon, in northern Alps (Chucholl *et al.*, 2015). Thus, notwithstanding the abundant literature on *A. pallipes* complex, a better knowledge of its distribution is needed. The boundaries of its native distribution are difficult to trace due to the spreading of crayfish by humans through voluntary introductions and the construction of new canals. Even if *A. pallipes* complex is non-indigenous in Sardinia, it deserves a *permit of residence* (Böhme, 2000), and should be protected from the several threats acting in the area, such as the progressive habitat degradation and loss, the increased human fishing and the spread of North American crayfish species

vector of the crayfish plague (*Procambarus clarkii* is already present in the adjacent Province of Olbia-Tempio; Holdich *et al.*, 2009; Piscia *et al.*, 2011). Genetic analyses, combined with ethological, ecological, and demographic studies (Brusconi *et al.*, 2008; Bertocchi *et al.*, 2008b), will help undertake the appropriate conservation actions.

CONCLUSIONS

The population of *Austropotamobius pallipes* complex in Sardinia belongs to the *A. i. meridionalis* subclade and, according to our findings, it seems to be healthy. Due to its *permit of residence*, as recommended by Cataudella *et al.* (2010) we encourage the protection and conservation of the local habitat of the nominate specimen to face any habitat degradation, increased human fishing as the spread of the North American crayfish, *Procambarus clarkii*.

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