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Preliminary data on the distribution, morphology and genetics of white-clawed crayfish and on their ectosymbionts in Lunigiana (Tuscany, Italy)

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Abstract – The white-clawed crayfish is a complex of species (*Austropotamobius pallipes* plus *A. italicus*, Crustacea) pivotal to riverine environments. Regrettably, it is endangered in many European countries due to a recent massive decline. We carried out a fine-scale survey on white-clawed crayfish distribution, morphology and genetics in an under-investigated region of central Italy (Lunigiana, Tuscany) to improve local knowledge and aid conservation management of the species. The torrential fauna of Lunigiana is scarcely known and habitat alterations due to the anthropic exploitation of the territory represent a potential threat to its persistence. We investigated crayfish occurrence in eight streams of the Magra River Basin. We performed nocturnal and diurnal transects, took morphometric measurements, collected samples for genetic analysis and checked for the occurrence of ectosymbionts. Crayfish were disclosed in two streams (Civasola, Verdesina). Morphometric and phylogenetic analysis (mtDNA COI gene Bayesian tree reconstruction) concurrently identified both populations as *Austropotamobius italicus carinthiacus*. In the Verdesina stream, crayfish were heavily infested by branchiobdellidans (Annelida). Verdesina crayfish were also significantly smaller than those found in the Civasola stream, where branchiobdellidans were absent. Hence, we hypothesized that such difference in size might be related to the high density of ectosymbionts. In the light of habitat features and of data herein provided, we propose that both Civasola and Verdesina streams should be considered as “natural ark sites” for *A. italicus*, with the Magra River Basin representing a regional stronghold for the conservation of the species.

Keywords: astacidae / branchiobdellida / cytochrome oxidase I / DNA barcoding / *Austropotamobius fulcivanus*

Résumé – **Données préliminaires sur la distribution, la morphologie et la génétique des écrevisses à pattes blanches et leurs ectosymbiotes en Lunigiana (Toscane, Italie).** L'écrevisse à pattes blanches est un complexe d'espèces (*Austropotamobius pallipes* plus *A. italicus*, Crustacé) qui est une espèce clé des environnements fluviaux. Malheureusement, elle est menacée dans de nombreux pays européens en raison d'un récent déclin massif. Nous avons réalisé une étude détaillée sur la distribution et la génétique de l'écrevisse à pattes blanches dans une région du centre de l'Italie (Lunigiana, Toscane) peu étudiée afin d'améliorer les connaissances locales et de faciliter la gestion de la conservation de l'espèce. La faune des rivières de Lunigiana est peu connue et l'altération des habitats due à l'exploitation anthropique du territoire représente une menace potentielle pour sa persistance. Nous avons étudié la présence d'écrevisses dans huit cours d'eau du bassin de la rivière Magra. Nous avons effectué des transects nocturnes et diurnes, pris des mesures morphométriques, prélevé des échantillons pour des analyses génétiques et vérifié la présence d'ectosymbiotes. Les écrevisses ont été trouvées dans deux cours d'eau (Civasola, Verdesina). L'analyse morphométrique et phylogénétique (reconstruction de l'arbre bayésien du gène de l'ADNmt COI) a permis d'identifier simultanément les deux populations comme *Austropotamobius italicus carinthiacus*. Dans le ruisseau Verdesina, les écrevisses étaient fortement infestées par les branchiobdellidés (Annelida). Les écrevisses de Verdesina étaient également significativement plus petites que celles trouvées dans le cours d'eau Civasola, où les branchiobdellidés étaient absents. Nous avons donc émis l'hypothèse que cette différence de taille pourrait être liée à la densité élevée des ectosymbiotes. À la lumière des caractéristiques

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de l'habitat et des données fournies ici, nous proposons que les deux cours d'eau Civasola et Verdesina soient considérés comme des “natural ark sites” pour *A. italicus*, le bassin de la rivière Magra représentant un bastion régional pour la conservation de l'espèce.

Mots-clés : astacidae / branchiobdellida / cytochrome oxydase I / code-barres d'ADN / *Austropotamobius fulcisanus*

1 Introduction

Crayfish are a very important component of freshwater habitats. Due to their role as detrital feeders and macrophyte grazers, they are pivotal to the population dynamics of both lentic and lotic waters, with their disappearance irremediably resulting in the alteration of the trophic net of a given ecosystem (Holdich and Reeve, 1991; Covich *et al.*, 1999; Scalici and Gibertini, 2005). In the light of their biological characteristics and the level of protection warranted across the whole of Europe, some crayfish are considered as good umbrella and/or flagship species for freshwater communities (Füreder and Reynolds, 2003; Scalici and Gibertini, 2005).

In the Western Palearctic, three genera of native freshwater crayfish occur: genus *Astacus* Fabricius 1775, with three native species, genus *Pontastacus* Bott, 1950, with nine species, and genus *Austropotamobius* Skorikow, 1907 (Crandall and De Grave, 2017). The latter has recently undergone a deep taxonomic revision and, at the present-time, includes three species: *A. torrentium* (Schrank, 1803), stone crayfish, *A. pallipes* (Lereboullet, 1858) and *A. italicus* (Faxon, 1914), which are both referred to as white-clawed crayfish. Recently, based on a study carried out by Manganelli *et al.* (2006), Crandall and De Grave (2017) referred to Italian white-clawed crayfish as *Austropotamobius fulcisanus fulcisanus* (Ninni, 1886). Nevertheless, in the present paper, in agreement with Fratini *et al.* (2005) and Bernini *et al.* (2016), we decided to use the more widespread binomial name *A. italicus*, as it is in line with the taxonomy in use in the National Center for Biotechnology Information (NCBI) database and, as such, with the phylogenetic reconstructions we have herein provided.

The distribution range of the three *Austropotamobius* species encompasses the Italian Peninsula (Fratini *et al.*, 2005; Trontelj *et al.*, 2005; De Luise, 2006; Morigio *et al.*, 2010; Kouba *et al.*, 2014; Jelic *et al.*, 2016), suggesting that Italy should be considered as a biodiversity hotspot for the genus (Baillie and Groombridge, 1996; Fratini *et al.*, 2005). More in detail, four subspecies are comprised within *A. italicus*: *A. i. carinthiacus*, *A. i. carsicus*, *A. i. italicus*, and *A. i. meridionalis* (Fratini *et al.*, 2005; Bernini *et al.*, 2016). Furthermore, *A. italicus* and *A. pallipes* are included in the *A. pallipes* species-complex, which is classified as Endangered by the International Union for the Conservation of Nature and Natural Resources and has been included in the EU Habitats Directive Annexes II/V and in the Appendix III of the Convention of Bern (Füreder *et al.*, 2010). Overall, in the last decades the *A. pallipes* species-complex populations have declined by 50–80% across Europe (Souty-Grosset and Reynolds, 2009). Major threats include occurrence of alien invasive crayfish species, spreading of diseases such as crayfish plague, poaching, overexploitation and habitat degradation (Füreder *et al.*, 2010). Declines can also occur locally as a result of water pollution and changes in the hydrological regime of rivers due

to anthropic use (Favilli and Manganelli, 2002; Legalle *et al.*, 2008; Füreder *et al.*, 2010).

Austropotamobius crayfish are generally considered sensitive to rapid environmental changes and water pollution. Despite their tolerance to broad variations of some parameters (Trouilhé *et al.*, 2007), the species of the *A. pallipes* complex seem to prevalently find suitable environmental conditions in rivers included in the class I of EBI (Extended Biotic Index) (Scalici and Gibertini, 2005) and to require good physico-chemical quality water, especially concerning oxygenation and pH (Jay and Holdich, 1977; Jay and Holdich, 1981; Trouilhé *et al.*, 2007; Beaune *et al.*, 2018). On the contrary, *A. torrentium* tolerates a broader range of water conditions (Svobodová *et al.*, 2012; Vlach *et al.*, 2012), although it can be impaired by organic pollution (Pârvolescu *et al.*, 2011). Nevertheless, *Austropotamobius* crayfish have been proposed as potential water quality bioindicators (Holdich and Reeve, 1991; Reynolds *et al.*, 2002) and the species of the *A. pallipes* complex are specifically employed for monitoring lotic ecosystems (Scalici and Gibertini, 2005).

Recently, researchers focused on occurrence, distribution and taxonomy of *A. pallipes* and *A. italicus* subspecies in Italy. *Austropotamobius pallipes* inhabits the north-western part of the Peninsula, whereas *A. italicus* occurs across all the remaining regions (Chiesa *et al.*, 2011), although it seems to be rarer in southern Italy and absent in Sicily and Sardinia (introduced stocks excluded: Aquiloni *et al.*, 2010; Amouret *et al.*, 2015). At the present-time, most studies refer to the Po River area (Trontelj *et al.*, 2005; Zaccara *et al.*, 2005; Ghia *et al.*, 2006), part of the Apennines (Trontelj *et al.*, 2005; Chiesa *et al.*, 2011), close to the borders with Austria and Slovenia (Trontelj *et al.*, 2005; Chiesa *et al.*, 2011) and, to a much lesser extent, to Liguria and Tuscany (Bertocchi *et al.*, 2008b; Chiesa *et al.*, 2011). Regrettably, many remote or poorly accessible areas have scarcely been investigated, such as, for example, the Lunigiana. Hence, the distribution range of *A. italicus* across Italy is still debated.

Lunigiana is a scarcely populated region (980 km²) located in the northern part of Tuscany (Fig. 1), whose relatively intact natural ecosystems include a large part of the mountainous and hilly tributaries of the Magra River.

In the last decades, private companies have built more than 20 Run-of-River (RoR) mini-hydroelectric plants (Paish, 2002; Douglas, 2007) in the secondary and tertiary tributaries of the Magra River, with others potentially in planning for the next future (Autorità Bacino Magra, 2000). Although such plants are often considered as low-impact when compared to the traditional ones (Paish, 2002; Kaunda *et al.*, 2012; Kern *et al.*, 2012; Lazzaro *et al.*, 2013; Gibeau *et al.*, 2016), it must be noticed that they cause alteration to water flow, water quality, and habitat degradation in the diversion reach (Douglas, 2007; Kumar and Katoch, 2015; Kern *et al.*, 2012; Lazzaro *et al.*, 2013; Gibeau *et al.*, 2016). Water removal for hydroelectric use and population isolation due to the

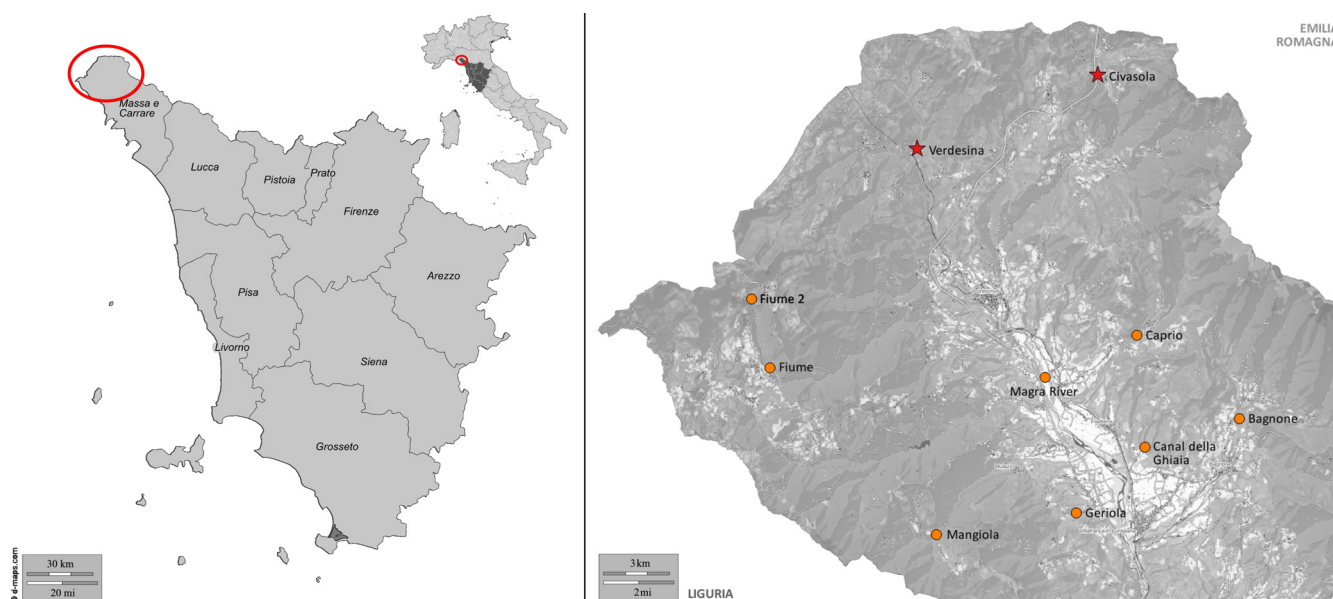


Fig. 1. Geographic context and map of the study area (Lunigiana, Tuscany, Italy). Nine transects were performed in eight streams. Red stars mark out the streams where we recorded white-clawed crayfish populations, while orange dots mark out surveyed streams where crayfish were not found. Modified from QGIS 2.14 open-source software (Quantum GIS Development Team, 2009).

presence of physical barriers in the streambed are generally considered to threaten the survival of both crayfish and aquatic invertebrates in general (Douglas, 2007; Pârvolescu and Zaharia, 2013; Stoch and Vigna Taglianti, 2014; Bologna *et al.*, 2016). Nevertheless, the physical barriers might possibly contribute to hinder the diffusion of invasive crayfish species (Kerby *et al.*, 2005; Frings *et al.*, 2013; Manenti *et al.*, 2014). Unfortunately, in the case of RoR plant construction, the lack of information on many protected species in the mountainous creeks of Lunigiana (crayfish included: Bertocchi *et al.*, 2008a; Ciuffardi *et al.*, 2009; Barbarotti *et al.*, 2012; Dini, 2015; Cianfanelli *et al.*, 2016) does not facilitate the implementation of good practice in land use and the enforcement of mitigation actions. Altogether, this seems to suggest that a deep investigation cannot be further delayed.

This study attempts to collect data on distribution and genetic identity of *A. italicus* in the upper part of the Magra River basin in Lunigiana and to highlight potential conservation issues of RoR plant installation. During this study, we also discovered and thus investigated the occurrence of branchiobdellidans in one of the detected crayfish populations.

2 Materials and methods

2.1 Study area

We selected eight secondary or tertiary tributaries of the Magra River, plus one site located in the upper part of the same watercourse (Tab. 1, Fig. 1). Streams were selected according to (in order of relevance): (i) preliminary surveys; (ii) location on both sides of the hydrographic basin; (iii) elevation above sea level; (iv) natural or partially modified streambed; (v) streambed accessibility.

Two streams, Civasola and Verdesina, were found to host white-clawed crayfish populations; hence, they are described here as focal areas of our study. Although both streams are

located under the municipality of Pontremoli, they largely differ from each other for some environmental features (Tab. 1). The Civasola stream is a right tributary of the Magra River; it originates in the Tuscan-Emilian Apennines and after 7 Km merges into the Magra River at locality “Molinello”. Its water quality is classified as in a “good” ecological and chemical state (Autorità di Bacino del Fiume Arno, 2017a). Our transect was located between 663 m (start) and 722 m (end) a.s.l.

The Verdesina stream originates in the Tuscan-Emilian Apennines at Monte Borraccia (1250 a.s.l.) and after 6 Km merges into the Verde stream, which is a right tributary to the Magra River in the town of Pontremoli. Its water quality is classified as in a “good ecological yet poor chemical state” (Autorità di Bacino del Fiume Arno, 2017b). Our transect started at 465 m a.s.l. and ended at 500 m a.s.l.

2.2 Data collection and morphological measurements

We carried out diurnal and nocturnal preliminary transects in all of the eight selected creeks, at least once a month, from May to September 2015 and from March to June 2016 (Tab. 1). We selected one transect per stream, with the length of each transect ranging between 200 and 500 m depending on riverbed accessibility. Due to logistic constraint, we assumed that the species was absent from a specific transect when we could not detect any *exuvia* nor living specimen after a minimum of two surveys.

For each transect, two operators walked counter-current on each side of the riverbed to geo-reference the occurrence of any crayfish or their remains (e.g., *exuviae*) using Garmin® GPS devices (Etrex 20x® and Etrex 30®). Whenever it was possible, potential shelters such as stones and underwater woods were flipped to check for hidden individuals. If many individuals were present in a small area (<5 m wide) such as side pools, they were geo-referenced as a single GPS point.

Table 1. Streams investigated in the present study, with municipality/locality, altitude on sea level of the starting point of each transect (**Alt**) and environmental features of the streams. **Av height** = average water height in the transect, during the sampling occasions. **Av width** = average width of the streambed within the transect. **Av substratum** = prevailing substratum in the riverbed, classified as megalithal (rocks and stones of >40 cm in diameter), macrolithal (stones between 20 and 40 cm in diameter), mesolithal (stones between 6 and 20 cm in diameter) and microlithal (small stones between 2 and 6 cm in diameter) (Buffagni and Erba, 2007). **Riparian vegetation** = presence of vegetation (trees and bushes) both autochthonous and allochthonous, along the banks of the stream. Streams hosting crayfish are highlighted in bold.

Stream	Municipality	Alt	Av height	Av width	Av substratum	Riparian Vegetation
Bagnone	Bagnone (Corlaga)	305 m a.s.l.	<30 cm	<10 m	Prevailing megalithal and macrolithal; very homogeneous	Present
Canal della Ghiaia	Villafranca in Lunigiana (Irola)	210 m a.s.l.	<30 cm	<3 m	Prevailing mesolithal and coarse organic material (dead leaves, mud, dead wood)	Present
Caprio	Filattiera (Rocca Sigillina)	345 m a.s.l.	<50 cm	<5 m	Highly prevailing megalithal, plus macrolithal	Present
Civasola	Pontremoli (Gravagna San Rocco)	663 m a.s.l.	<20 cm	< 3 m	Prevailing microlithal, coarse organic material (dead leaves, mud, dead wood) and artificial substratum	Present
Fiume	Zeri (Noce di Zeri)	595 m a.s.l.	<20 cm	<10 m	Prevailing mesolithal and macrolithal; very homogeneous	Scarce
Fiume2	Zeri (Villaggio degli Aracci)	1095 m a.s.l.	<50 cm	<5 m	Prevailing mesolithal and macrolithal	Present
Geriola	Mulazzo (Stallone-Talavorno)	205 m a.s.l.	<20 cm	<10 m	Prevailing macrolithal; very homogenous	Present
Mangiola	Mulazzo (Montereggio)	470 m a.s.l.	<60 cm	<5 m	Prevailing megalithal and macrolithal	Present
Magra	Pontremoli (Pracchiola)	650 m a.s.l.	<20 cm	<10 m	Prevailing megalithal and dead wood	Present
Verdesina	Pontremoli (Guinadi)	465 m a.s.l.	<60 cm	<5 m	Prevailing megalithal and macrolithal	Present

From June to September 2016, we only investigated Civasola and Verdesina streams, performing three additional nocturnal surveys per stream (one visit per month). As such, we performed a total of 5 surveys on Civasola stream and 11 on Verdesina stream. One pereopod (non-lethal sample) selected at random for each manipulated crayfish was cut with blades, sterilised by 1-minute dipping in a 2.5% sodium hypochlorite solution, and then preserved in 96% ethanol before being stored at -32°C for genetic analysis. We sampled 48 (21 males, 26 females, 1 undetermined) and 44 (22 males, 16 females, 6 undetermined) individuals in the Civasola and Verdesina, respectively. We measured (i) cephalothorax length (CL), from the rostral apex to the posterior median edge of the cephalothorax, to determine class age and sexual maturity of the individuals (Pratten, 1980; Scalici and Gibertini, 2009; Scalici and Gibertini, 2011; Ghia *et al.*, 2015; Wendler *et al.*, 2015), (ii) rostrum total length (RL) and (iii) length of the rostrum apex (AL) to calculate AL/RL *ratio*, in order to obtain a preliminary morphological identification of the species (Grandjean *et al.*, 2000a; Bertocchi *et al.*, 2008a; Bertocchi *et al.*, 2008b; Chucholl *et al.*, 2015). All parameters were measured to the nearest 0.1 mm using a slide calliper (Mitutoyo[®] 530 series); the crayfish were then released at the catching site. Additionally, we collected and dry-preserved all visible *exuviae* to take the same biometric measurements also from intact carapaces.

Only crayfish found in the Verdesina stream hosted branchiobdellidans on cephalothorax, chelae and/or pereopods. In the field, we visually estimated, but did not manually count, the density of ectosymbionts according to three different

abundancy classes: low abundancy of ectosymbionts (no visible worms on crayfish surface), medium (approximately less than 20 worms per crayfish) or high (more than 20 worms per crayfish).

2.3 Statistical analysis on morphometric data

Analyses were performed with statistical software RStudio[®] Desktop 1.0.143 (RStudio Team, 2015). First, we checked for normal distribution and homoscedasticity of morphometric variables with graphical representations, Shapiro-Wilk test and Bartlett's test. Sex *ratio* was calculated from manipulated individuals as the proportion of males relative to females. Since data were normally distributed, two-way ANOVA was performed on AL/RL *ratio*/sex/stream relations and on CL/sex/stream relations, with subsequent Tukey's honest significance post-hoc test. Sex *ratio* among streams was tested with Pearson's Chi-squared test. Level of ectosymbiont occurrence between streams was tested with non-parametric Kruskal-Wallis rank sum test. Subsequently, we calculated Spearman r_s and then performed one-way ANOVA on cephalothorax length/level of ectosymbiont occurrence relations, followed by Tukey's honest significance post-hoc test. Finally, ectosymbiont prevalence between sexes was tested with the non-parametric Kruskal-Wallis rank sum test. Given that the parasitized individuals with a CL > 45.0 mm were all males, we performed this test only on crayfish with a CL ranging between 25.0 and 45.0 mm, to include individuals of both sexes and comparable size.

2.4 DNA extraction, amplification and sequencing

Genomic DNA was extracted from six randomly selected pereopods (Civasola, N=3; Verdesina, N=3). We used Genra® Puregene® Core Kit-A (Qiagen, Germany) following the manufacturer's instructions. The reliability of each extraction was checked through multiple negative controls. DNA concentration and purity were assessed with an Eppendorf BioPhotometer (AG Eppendorf).

We amplified a 1,173 bp-long portion of the mitochondrial DNA (mtDNA) gene coding for the subunit I of the Cytochrome Oxidase (COI) using primers FC_COI5'-F (5'-TTTGGCACTTGAGCTGGGATAG-3') and FC_COI3'-R (5'-GCATCTGGATAATCAGAATACC-3') (Bernini *et al.*, 2016). PCRs were run in a MyCycler™ thermal cycler (Biorad, USA) with the following thermal profile: 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, followed by 7 min at 72 °C. Reactions (50 µl) were prepared with 1 µl of AmpliTaq Gold DNA Polymerase (1 U/µl, Thermo Fisher Scientific), 4 µl of 25 mM MgCl₂ (Thermo Fisher Scientific), 5 µl of 10X PCR Gold buffer (Thermo Fisher Scientific), 5 µl of 2.5 mM dNTPs (Sigma Aldrich), 3 µl of each primer (1 µM) and 20 ng of DNA template. PCR products were purified (Genelute PCR Clean-up Kit, Sigma Aldrich) and directly sequenced on both DNA strands using internal primers (A.ital_COI_644Fw: 5'-CTTCATTTTTTGGATCCYGGCTGG-3' and A.ital_COI_898Rev: 5'-GTAGCAGAAGTAAAA-TATGCTCG-3'; GATC Biotech, Germany). We performed the alignment with Clustalx (v. 1.81; Thompson *et al.*, 1997) using a 534 bp-long fragment (from pos. 118 to pos. 651 of *A. pallipes* NC026560; Grandjean *et al.*, 2016; codon reading frame, 1) plus 114 GenBank sequences of *Austropotamobius* subspecies (Supplementary Table 1). *Austropotamobius pallipes* NC026560 was used as outgroup (Grandjean *et al.*, 2016).

All sequences were deposited at the National Centre for Biotechnology Information (GenBank accession codes: MG244267- MG244272, Supplementary Table 1).

2.5 Mitochondrial DNA analyses

We used arlequin (v. 3.5.1; Excoffier and Lischer 2010) to infer haplotypes and check for neutral evolution of the mtDNA sequences (Tajima's *D*: Tajima, 1989). We used Smart Model Selection (SMS) (Lefort *et al.*, 2017) as implemented in PhyML 3.0 (Guindon *et al.*, 2010) and found that the HKY (Hasegawa *et al.*, 1985) + G ($\alpha = 1.361$) + I (=67.5%) was the best evolutionary model fitting to our crayfish dataset according to both the Akaike (AIC=4,047.6) and Bayesian (BIC=5,378.8) Information Criterion. In a Bayesian (BI) analysis, however, Metropolis-coupled Monte Carlo Markov Chains integrate over the uncertainty in parameter values. Hence, only the general form of the model was included in the BI performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Two independent runs of analysis were conducted for 3 500 000 generations with a sample frequency of 100 (four chains, heating=0.2, random starting tree). Convergence between runs was monitored through the Average Standard Deviation of Split Frequencies (ASDSF) until this value dropped well below 0.01. Stationarity was reached after 700 000 generations (ASDSF=0.006414) as inferred using Tracer

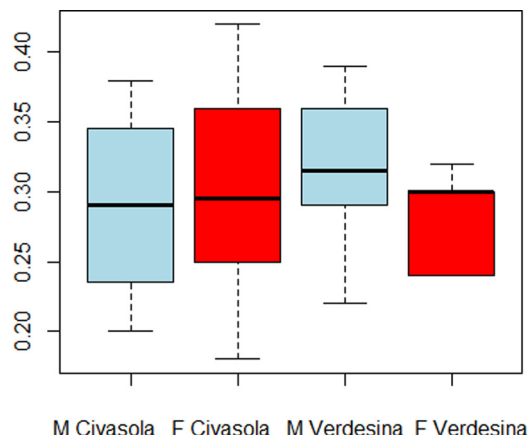


Fig. 2. Boxplot of the *ratio* between the length of rostrum tip (apex length, AL) and the total rostrum length (RL), calculated according to sex and stream (males in light blue, females in red) with median, interquartile range and minimum/maximum values of the parameter.

1.5.0 (Rambaud and Drummond 2007). Hence, 14 000 trees were discarded as burn-in, and the remaining 56 002 trees were used to produce a 50% majority-rule consensus tree.

3 Results

3.1 *Austropotamobius italicus*

Two out of the eight investigated streams hosted white-clawed crayfish: Civasola and Verdesina (Tab. 1). In all the other streams, we did not record any living specimen nor any sign of presence (*exuviae*, burrows, etc.).

Sex *ratio* was males:females 0.81 (N=47) and 1.38 (N=38) for Civasola and Verdesina stream, respectively, with the difference being not statistically significant (χ -squared = 2.26, *df*=1, *p*=0.13). Preliminary morphological analysis based on AL/RL *ratio* (mean: 0.31 and SD: 0.06, N=92) assigned the individuals to the *A. italicus* clade. The AL/RL *ratio* values were also analysed individually for streams and sex and differences were not statistically significant in either case (*p* > 0.10, Fig. 2).

As for the cephalothorax length (CL), males were significantly bigger than females in both streams, but both sexes were bigger in Civasola than in Verdesina (*p* < 0.05 for "sex" factor, *p* < 0.001 for "stream" factor, see Fig. 3). In the Civasola, CL ranged between 29 and 61 mm in males (N=20, mean: 46.3 mm and SD: 9.8 mm) and between 32 and 54 mm in females (N=25; mean: 42.0 mm and SD: 5.3 mm), while in the Verdesina between 26 and 56 mm in males (N=22; mean: 38.3 mm and SD: 7.1 mm) and between 22 and 44 mm in females (N=16; mean: 33.5 mm and SD: 6.8 mm). Only 3.1% of our records showed a CL < 25 mm.

Only one COI mtDNA haplotype (H31) was found in the Civasola and Verdesina streams. Overall, the alignment included 63 haplotypes (outgroup included) with 97 variable sites: among these, 71 were parsimoniously informative. The 121 mtDNA aligned sequences showed G-biased (21.5%) nucleotide composition, high Ti/Tv *ratio* (34.4), did not contain any internal stop codon/indels, and they conformed to the neutral model of evolution (Tajima's *D* = -0.254, *p* > 0.05).

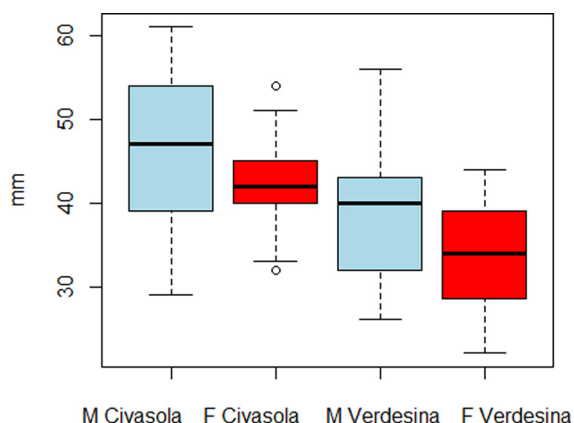


Fig. 3. Boxplot of cephalothorax lengths (mm) calculated according to sex and stream (males in light blue, females in red), with median, interquartile range and minimum/maximum values of the parameter.

Phylogenetic reconstructions marked out the occurrence of three clades: the first included *A. i. meridionalis* (PP = 1.0), the second *A. i. carsicus* (PP = 0.63), and the third *A. i. italicus* with *A. i. carinthiacus* (PP = 1.0). Lunigiana haplotype H31 was assigned to *A. i. carinthiacus* subspecies (PP = 1.0) and it was shared with 11 representatives from the northern slope of the Apennines (Fig. 4, Supplementary Table S1).

3.2 Branchiobdellidan worms

Branchiobdellidan worms (Annelida, Clitellata) were genetically identified as *Branchiobdella italica* Canegallo, 1929 (mtDNA COI genotyping: Boschetti *et al.*, in prep.). The occurrence of branchiobdellidans was highly significantly different between streams (N = 85, *d.f.* = 1, $p < 0.001$); indeed, in the Civasola stream, branchiobdellidans were not found. In the Verdesina stream, 81.9% of manipulated crayfish (N = 44: 90.9% of males and 75.0% females) hosted branchiobdellidans. Crayfish CL positively correlated with density of branchiobdellidans (N = 38, Spearman $r_s = 0.81$, *d.f.* = 2, $p < 0.001$). This difference was highly statistically significant for both high *vs.* low and high *vs.* medium level of worm density (N = 38, *d.f.* = 2, $p < 0.001$), and statistically significant for medium *vs.* low level (N = 38, *d.f.* = 2, $p < 0.01$). When the difference in the prevalence of branchiobdellidans in males and females was tested, the outcome was not statistically significant (N = 30, *d.f.* = 1, $p > 0.05$).

4 Discussion

The occurrence of *A. italicus carinthiacus* in the mountainous part of the right slope of the Magra River Basin was confirmed; nonetheless, the species appears to be uncommon and the studied populations could be relevant for the conservation of the species in the area.

4.1 Species occurrence and distribution

Literature records reporting on wildlife occurrence and distribution in Lunigiana is scant. This made the selection of

streams for crayfish investigation a hard task. Civasola and Verdesina are located close to the SAC IT4020020, where *A. italicus* had been previously reported (Barbarotti *et al.*, 2012). In both streams, the most individuals were found outside the main water flow, i.e. in pools located near the banks or waterfalls where the speed of the current is quite low. Almost all sampled crayfish hold cephalothorax length proper to adult individuals (Scalici and Gibertini, 2011; Ghia *et al.*, 2015; Wendler *et al.*, 2015; see also below). However, this result might not reflect a real disequilibrium in the distribution of the age classes. Indeed, visual search by transects more easily detect adults rather than young individuals, with the hatchlings being usually underestimated (Peay, 2003; Wendler *et al.*, 2015). Hence, the prevailing occurrence of adults might have suffered from a bias in the methods and did not come as a surprise.

4.2 Subspecies identification

In recent years, several authors investigated distribution, phylogenetic relationships and taxonomical placement of *A. italicus* in Italy and Europe. Our study area was almost uninvestigated. However, data from nearby regions (Liguria, Emilia-Romagna, Tuscany itself) suggested the potential occurrence of either *A. i. italicus* or *A. i. carinthiacus*, with *A. i. meridionalis*, *A. i. carsicus* and *A. pallipes* representing a much rarer eventuality (Fratini *et al.*, 2005; Cataudella *et al.*, 2006; Bertocchi *et al.*, 2008a; Cataudella *et al.*, 2010; Chiesa *et al.*, 2011). In this study, the AL/RL ratio assigned both crayfish populations to *A. italicus*, since its value was much closer to the ones known for this species, than to the ones reported for *A. pallipes*. Indeed, Laurent and Suscillon (1962) and Grandjean *et al.* (1998) reported an AL/RL ratio value of respectively 0.32 and 0.29 for *A. italicus* and 0.20 and 0.22 for *A. pallipes*, those values being confirmed by Chucholl *et al.* (2015).

Due to the high morphological variability occurring at the intraspecific level (Ghia *et al.*, 2006; Bertocchi *et al.*, 2008a), a DNA barcoding was also carried out to achieve a more reliable taxonomic identification. As shown in Figure 4 and Supplementary Table 1, we proved that both crayfish populations were assigned to the taxon *A. i. carinthiacus*, thus expanding the available distribution range of this subspecies in central Italy. The unique haplotype found in Lunigiana was shared by individuals from Lombardia (8), Emilia-Romagna (2) and Tuscany (1) (Fig. 4). Nevertheless, it is worth recalling that several authors (Machino, 1997; Holdich, 2002; Cataudella *et al.*, 2006; Jelic *et al.*, 2016) suggested that *A. i. italicus* and *A. i. carinthiacus* should be included in the same subspecies because of their limited genetic and morphological divergence; our reconstruction, which assigned *A. i. carinthiacus* to the *A. i. italicus* clade (Fig. 4), is in perfect agreement with these authors.

4.3 Sex ratio and sexual maturity

The sex ratio of both populations was unbalanced, even though the values did not significantly differ between the Civasola and Verdesina streams. Several studies reported a balanced sex ratio for *A. pallipes* species complex populations (Nowicki *et al.*, 2008; Brusconi *et al.*, 2008; Wendler

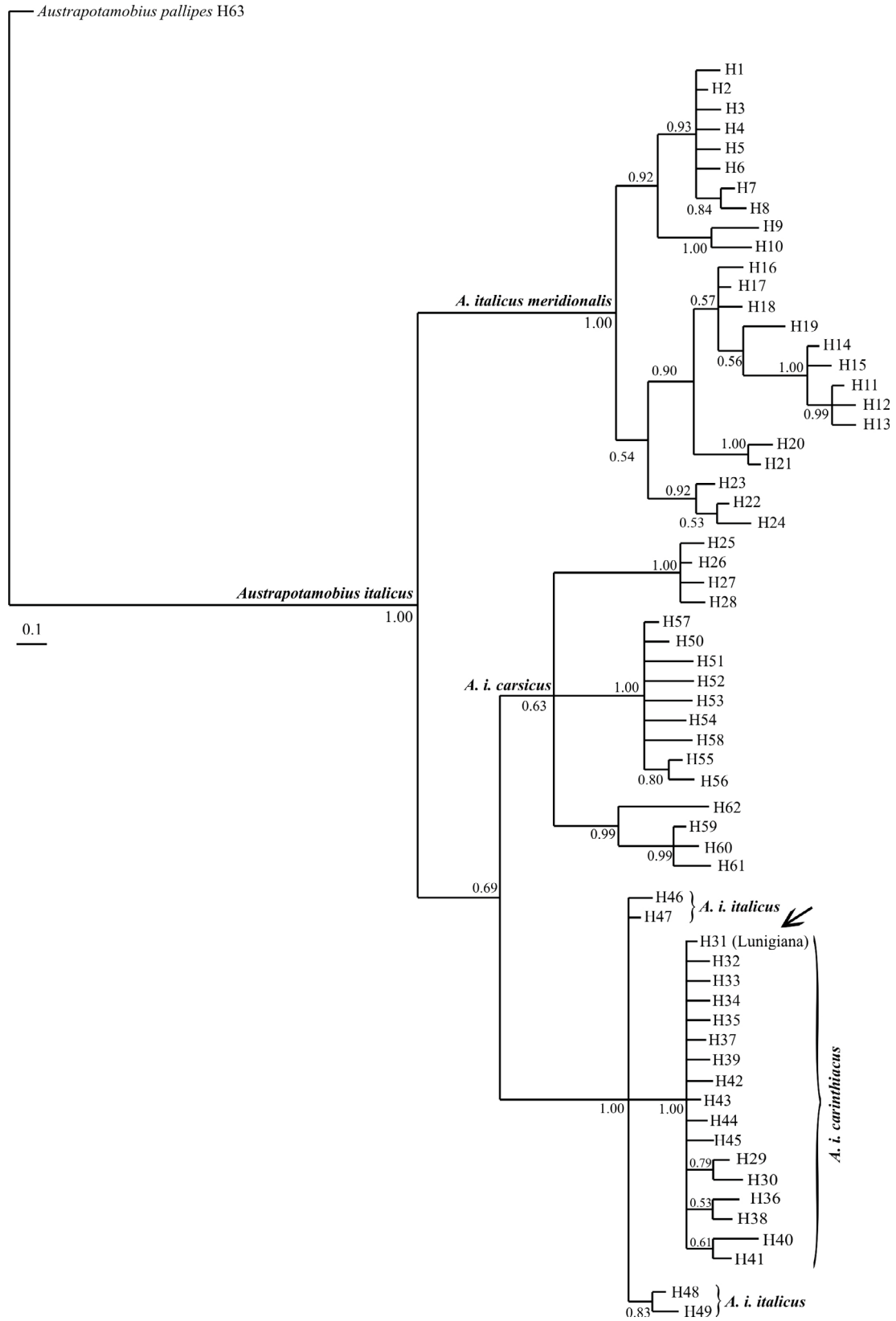


Fig. 4. Bayesian (BI) tree computed on crayfish individuals using 62 COI haplotypes (H, 534 bp-long sequence alignment) and *A. pallipes* NC026560 as outgroup (Supplementary Table 1). The statistic support was reported at each node. Lunigiana samples (N = 6) are identified by haplotype H31.

et al., 2015). Nevertheless, some cases of biased sex ratio in healthy *Austropotamobius* populations are also known (Grandjean *et al.*, 2000b; Scalici and Gibertini, 2005), up even to a males:females sex ratio of 1:1.9 (Grandjean *et al.*, 2000b). Therefore, our data might either fall within the limits of the demographic variability of the species or depend on the limited sample size available for our populations.

Although in the *Austropotamobius* genus a significant differentiation among populations from different localities is known to occur, a 25 mm-long cephalothorax usually represents the minimum size in sexually mature white-clawed crayfish (Scalici and Gibertini, 2011; Ghia *et al.*, 2015; Wendler *et al.*, 2015; Dakic and Maguire, 2016; Maguire *et al.*, 2017), with females usually reaching sexual maturity at a smaller size than males (Grandjean *et al.*, 1997). Therefore, we considered all our sampled individuals as sexually mature. Sexual dimorphism and sex-specific growth pattern are known to occur in the *Austropotamobius* genus (Bertocchi *et al.*, 2008a; Scalici and Gibertini, 2009; Scalici *et al.*, 2010b; Vlach and Valdmanová, 2015). We confirmed that males usually have longer cephalothorax than females (Vlach and Valdmanová, 2015; Wendler *et al.*, 2015).

4.4 Morphometry and ectosymbionts

For both sexes, the Civasola crayfish showed, on average, longer cephalothorax than the Verdesina ones. For instance, the longest individual (male, Civasola) showed a 61 mm-long cephalothorax, a value much higher than those reported in the literature for *A. italicus* and strictly related species (Matthews and Reynolds, 1995; Streissl and Hödl, 2002; Scalici *et al.*, 2010b; Scalici and Gibertini, 2011; Caprioli *et al.*, 2014; Vlach and Valdmanová, 2015). It could be hypothesized that such difference in cephalothorax length might be related to environmental factors (e.g., difference in food availability of Civasola and Verdesina streams) or to the age structure of the two populations (e.g., younger crayfish have a smaller CL). Nevertheless, the occurrence of Branchiobdellida in only one of the two streams deserves some attention. Branchiobdellidans are obligate ectosymbionts of crayfish and, depending on different environmental conditions, they can act as commensals, mutualists or parasites (Scalici *et al.*, 2010a; Brown *et al.*, 2012; DeWitt *et al.*, 2013; Skelton *et al.*, 2013; Vedia *et al.*, 2014; Skelton *et al.*, 2016; Vedia *et al.*, 2016). Some species of branchiobdellidans can positively influence their hosts' body size and mass (Keller, 1992; Brown *et al.*, 2002; DeWitt *et al.*, 2013; Vedia *et al.*, 2016). On the contrary, other species, when occurring at high density, can show parasite habits by consuming host tissues and thus affecting crayfish growth (Scalici *et al.*, 2010a; Brown *et al.*, 2012; Skelton *et al.*, 2013; Skelton *et al.*, 2016). On average, branchiobdellidan worms occurred at high density on Verdesina crayfish, which showed smaller CL values when compared to those from the Civasola stream. Although we cannot rule out other hypotheses, such as those above mentioned, we tentatively suggest that *B. italyca* could act as parasite on *A. italicus*, with the abundance of branchiobdellidans either directly affecting host growth (Scalici *et al.*, 2010a; Brown *et al.*, 2012; Skelton *et al.*, 2013; Skelton *et al.*, 2016) or indirectly decreasing the fitness of the largest individuals.

Branchiobdellidan worms tend to occur more frequently on large crayfish: on the one hand, an increase in size can allow for a higher colonization of epizoic organisms; on the other hand, large crayfish moult less often than small ones (Brown *et al.*, 2002; Brown *et al.*, 2012; DeWitt *et al.*, 2013; Vedia *et al.*, 2016). Accordingly, we found, on average, that individuals with a longer cephalothorax were more heavily parasitized than those with a shorter one. As previous studies have shown (Vedia *et al.*, 2016), the prevalence of branchiobdellidans between sexes did not significantly differ, when analysing individuals of comparable size (CL ranging between 25.0 and 45.0 mm), thus confirming that the sex factor *per se* does probably not influence branchiobdellidan occurrence.

4.5 Local conservation issues

In Tuscany, mountainous or hilly streams are particularly valuable for the conservation of *A. italicus*, as they can act as “ark sites” (Holdich *et al.*, 2004; Souty-Grosset and Reynolds, 2009; Nightingale *et al.*, 2017; Rosewarne *et al.*, 2017) in the light of the occurrence of the invasive *Procambarus clarkii* Ortmann, 1905 (red swamp crayfish). Red swamp crayfish have largely contributed to the decline of *A. italicus* in central Italy through habitat competition and pathogen transmission (Legalle *et al.*, 2008), since *P. clarkii* introduction in Tuscany in the '90s (Barbaresi and Gherardi, 2000). The distribution range of this invasive exotic species seems limited by elevation (Gil-Sánchez and Alba-Tecedor, 2002); therefore, high altitude streams could be fundamental for the survival of autochthonous *A. italicus* in the upper part of watercourses colonized by *P. clarkii*.

The two streams identified in the present study harbour viable populations of *A. italicus* and meet the main criteria for the definition of ark sites. Thus, we suggest that the Civasola and Verdesina streams could be considered as “natural ark sites” for the conservation of the species in Tuscany and deserve an adequate protection level.

RoR plants, although less impacting than traditional ones, cannot prevent habitat stresses for aquatic invertebrates and may have cumulative effects when multiple RoR projects exist on the same river basin (Douglas, 2007; Kern *et al.*, 2012; Lazzaro *et al.*, 2013; Gibeau *et al.*, 2016). Thus, a proper and updated knowledge of local torrential fauna is mandatory to consider the occurrence of protected species (Douglas, 2007) and to allow a careful evaluation of a potentially detrimental use of the territory.

5 Conclusions

The insufficient knowledge of the local biodiversity heritage can lead to a lack of proper conservation and management actions or even to harmful over-exploitation. We have contributed to the update of *A. italicus* distribution and to the knowledge of morphological and genetic characteristics of central Italy populations, and we have gained an insight into a potential parasitic role of *B. italyca* on *A. italicus* that deserves future investigation. Further studies will be required in order to develop proper conservation plans for *A. italicus* in the Lunigiana region.

Supplementary Material

Supplementary Table S1.

The Supplementary Material is available at <https://www.kmae-journal.org/10.1051/kmae/2018013/olm>.

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