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RESEARCH ARTICLE

Complexity of biogeographic pattern in the endangered crayfish *Austropotamobius italicus* in northern Italy: molecular insights of conservation concern

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Abstract The protection of freshwater biodiversity has become a priority task for conservation practices, as freshwater ecosystems host high levels of cryptic diversity, while also record similarly high rates of extinction. The Italian white-clawed crayfish *Austropotamobius italicus* is an endemic freshwater crustacean, threatened by several anthropogenic impacts such as habitat fragmentation, pollution, invasion of exotics, and climate change. Previous phylogenetic studies conducted in Italy pointed out a complex phylogeographic framework for the species, with four different subspecies currently recognized. Conservation efforts, particularly when involving restocking and reintroduction, require a detailed knowledge of their population genetics. In this study we describe the genetic structure of *A. italicus* populations in northern Italy (Lombardy Alpine foothills and northern Apennines) by using the informative mitochondrial marker cytochrome *c* oxidase subunit I, in order to assess their current evolutionary diversity and past phylogeographic history from a conservation perspective. Our results contribute to the mapping of the contact area among *A. i. carsicus* and *A. i. carinthiacus* in the Orobie Larian Prealps. More interestingly, we highlight the existence of two deeply differentiated evolutionary lineages within *A. i. carsicus*, showing alternative phylogeographic patterns and past demographic trends. We propose to consider these two clades as distinct molecular operational taxonomic units for the conservation of this endangered crayfish.

Keywords *COI* mtDNA · Phylogeography · Alps · Apennines · *Austropotamobius pallipes* complex · MOTU

Introduction

With less than the 1 % of the Earth surface supporting nearly 10 % of all known species, freshwater biotopes host some of the richest and most diverse ecosystems on the planet, but also the most endangered ones (Abramovitz 1996; McAllister et al. 1997; Revenga and Mock 2000; Dudgeon et al. 2006; Balian et al. 2008). Extinctions occur at higher and faster rates in freshwater biotopes than in any other environment because of overexploitation, water pollution, flow modification, destruction or degradation of habitat, and invasion by exotic species, all together severely affecting the persistence of autochthonous species (Ricciardi and Rasmussen 1999; Malmqvist and Rundle 2002; Jenkins 2003; Dudgeon et al. 2006). As a result, nowadays the conservation of freshwater biodiversity has become a key issue for the worldwide scientific community (e.g. Saunders et al. 2002; Strayer and Dudgeon 2010; Martinuzzi et al. 2014). Moreover, the adoption of innovative molecular approaches is rapidly coming into favour to disclose previously unknown biological diversity, and cryptic species that occur in freshwater ecosystems (e.g. Baker et al. 2003, 2004; Chenoweth and Hughes 2003; Perdices et al. 2005; Cook et al. 2006; Page and Hughes

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2007; Mathews et al. 2008; Dawkins et al. 2010). Thus, it is essential to apply correct conservation policies, to account for high levels of hidden genetic variation and avoid the loss of undetected biological richness (Margules and Pressey 2000; Cook et al. 2008).

In freshwater crustaceans, cryptic variation has been repeatedly pointed out by the analysis of molecular polymorphisms at the mitochondrial gene encoding for the cytochrome *c* oxidase subunit I (COI) (Zaccara et al. 2005; Witt et al. 2006; Apte et al. 2007; Bentley et al. 2010; Dawkins et al. 2010; Filipová et al. 2010; Chiesa et al. 2011; Klobučar et al. 2013). Since genetic variability plays a crucial role in shaping the evolutionary paths for populations and species (e.g. by promoting organism adaptation to environmental changes, Avise 2000; Moritz et al. 2002), the detection of alternative evolutionary significant units (ESUs, Moritz 1994; Fraser and Bernatchez 2001) or molecular operative taxonomical units (MOTUs, Floyd et al. 2002) is the fundamental key for planning long-term conservation actions, particularly when dealing with endangered species. As threatened freshwater crayfish generally show limited dispersal abilities, the achievement of a detailed molecular framework for these species represent the only way to ensure the preservation of local independent gene pools, especially in those regions (i.e. hot-spots) already known to be the natural repositories of the species' genetic diversity.

The Italian white-clawed crayfish *Austropotamobius italicus* (Faxon 1914) is a freshwater crustacean, ascribed to the *Austropotamobius pallipes* (Lereboullet 1858) species complex (Souty-Grosset et al. 2006). Once its geographical range was wide in Europe (Souty-Grosset et al. 2006), in recent years it has been drastically reduced (Holdich et al. 2009) so that the species from 2010 has been classified as "endangered" in the "IUCN Red List of Threatened Species" (Füreder et al. 2010); it has also been listed under the EU Habitats Directive 92/43/EEC (Annexes II and V) and therefore requires the designation of special areas of conservation for its protection. Indeed, its populations have severely suffered in the past decades from competition with exotic crayfish (Vorburger and Ribi 1999; Gherardi 2006; Gherardi et al. 2013) such as *Orconectes limosus* (Rafinesque 1817), *Procambarus clarkii* (Girard 1852) and *Pacifastacus leniusculus* (Dana 1852), and from deadly epidemics caused by *Aphanomyces astaci* Schikora 1906, an oomycete responsible for the so-called "crayfish plague" introduced since 1850′s in the Po river basin (Alderman 1996; Morpurgo et al. 2010). Other anthropogenic factors, including climate change, pollution, habitat destruction and fragmentation, and water depletion for agriculture and industry, have further accelerated the decline of the native white-clawed crayfish all over its range (Nardi et al. 2005; Aquiloni et al. 2010; Füreder et al. 2010). Thus, residual populations of the *Austropotamobius pallipes* complex are now confined to higher elevations, where the exotic crayfish have not yet expanded, and the habitat is less influenced by human activities (Changeux 2003; Ghia et al. 2013). These populations are now isolated reproductive units, often confined to single mountain streams or to separate basins.

Isolated small populations, such as those resulting from demographic bottlenecks, are more likely to experience the negative effects of stochastic genetic drift. These conditions, in general lead to a loss of intra-population genetic diversity and to increased inter-population diversity, which may both negatively affect their long-term viability (Wright 1978). Under this metapopulation scenario, the lack of adequate knowledge on the distribution of the genetic structure may hinder the conservation and management efforts for endemic species (Brito 2004). A number of evolutionarily significant units (ESUs, Crandall et al. 2000) have previously been identified within the white-clawed crayfish in France (Gouin et al. 2006), and the Iberian Peninsula (Diéguez-Uribeondo et al. 2008). In Italy, recent studies based on mitochondrial DNA (mtDNA) highlighted a complex phylogeographic framework for *A. italicus*, with four different subspecies currently recognized across the Italian peninsula: *A. i. carinthiacus* in central and north-western Italy; *A. i. carsicus* in north-eastern Italy; *A. i. italicus* on the Tuscan-Emilian Apennines (central Italy); *A. i. meridionalis* in southern Italy (Fratini et al. 2005).

Conservation efforts of this species in Lombardy are contemporarily challenged by anthropogenic factors (Lombardy has ten million inhabitants for a mean density of 417 inhab./km^2) and a highly complex genetic population structure due to a possible contact zone between *A. i. carinthiacus* and *A. i. carsicus* in the Orobie Alps (Fratini et al. 2005). Identifying the contact zone between the two subspecies is particularly crucial for the conservation and maintenance of the species biodiversity. Indeed in the past decades some conservation projects, aimed to protect threatened populations of native crayfish in Lombardy, have led to restocking and reintroduction plans acquiring substantial data on the population genetic structure (conservation projects supported by EU's financial instrument, e.g. LIFE00 NAT/IT/7159, LIFE03 NAT/IT/000147, and LIFE08 NAT/IT/000352). Future conservation programmes aimed at conserving genetic diversity cannot be undertaken without a thorough knowledge of taxonomic entities, particularly in zones where subspecies overlap (Souty-Grosset and Reynolds 2009).

Our work aimed to genotype the residual white-clawed crayfish populations in the Lombardy Alpine foothills and the northern Apennines, and in particular: (1) to assess their phylogeny with respect to the mitochondrial clades/subspecies

previously identified for the species; (2) to identify the contact zone between *A. i. carsicus* and *A. i. carinthiacus*; (3) to investigate the level of genetic variability among the residual populations; (4) to identify new units of operational management for conservation. Results from this study should allow funds and efforts be more appropriately addressed for the conservation of genetic biodiversity in the residual populations of this endangered species.

Materials and methods

Population sampling and laboratory procedures

A total of 205 *A. italicus* crayfish were collected by hand at 53 localities, distributed along 39 Alpine streams ($n = 158$ samples) and 14 Apennine streams ($n = 47$ samples), belonging to ten hydrographic basins within the Po river basin (Fig. 1; see Online Resource 1 for location data). Tissue samples were taken by removing a small portion of a pereopod from each crayfish, and by preserving it in ethanol at −20 °C. This procedure, commonly used in genetic study of crayfish, avoids sacrificing the individual, whose biological activities remain unaffected. Moreover, pereopods are easily regenerated in decapods at the next moult.

Total DNA extraction was performed using the commercial NucleoSpin®Tissue kit (Macherey-Nagel, Düren, Germany), following manufacturer's instructions. Genomic DNA was stocked at −20 °C. Mitochondrial gene encoding for cytochrome *c* COI is a valuable marker to analyze the genetic variation at the intraspecific level, in crayfish and other crustaceans (as confirmed by several authors, e.g. Meyran et al. 1997; Versteegen and Lawler 1997; Meyran and Taberlet 1998; Haye et al. 2004; Shull et al. 2005). Moreover, the effect of stochastic drifts, such as those resulting from demographic bottlenecks, has greater effects on mitochondrial than nuclear genes (the former being uniparentally inherited without recombination, Ovenden and White 1990). Therefore, we focus on mtDNA in order to investigate both past phylogeographic patterns that have

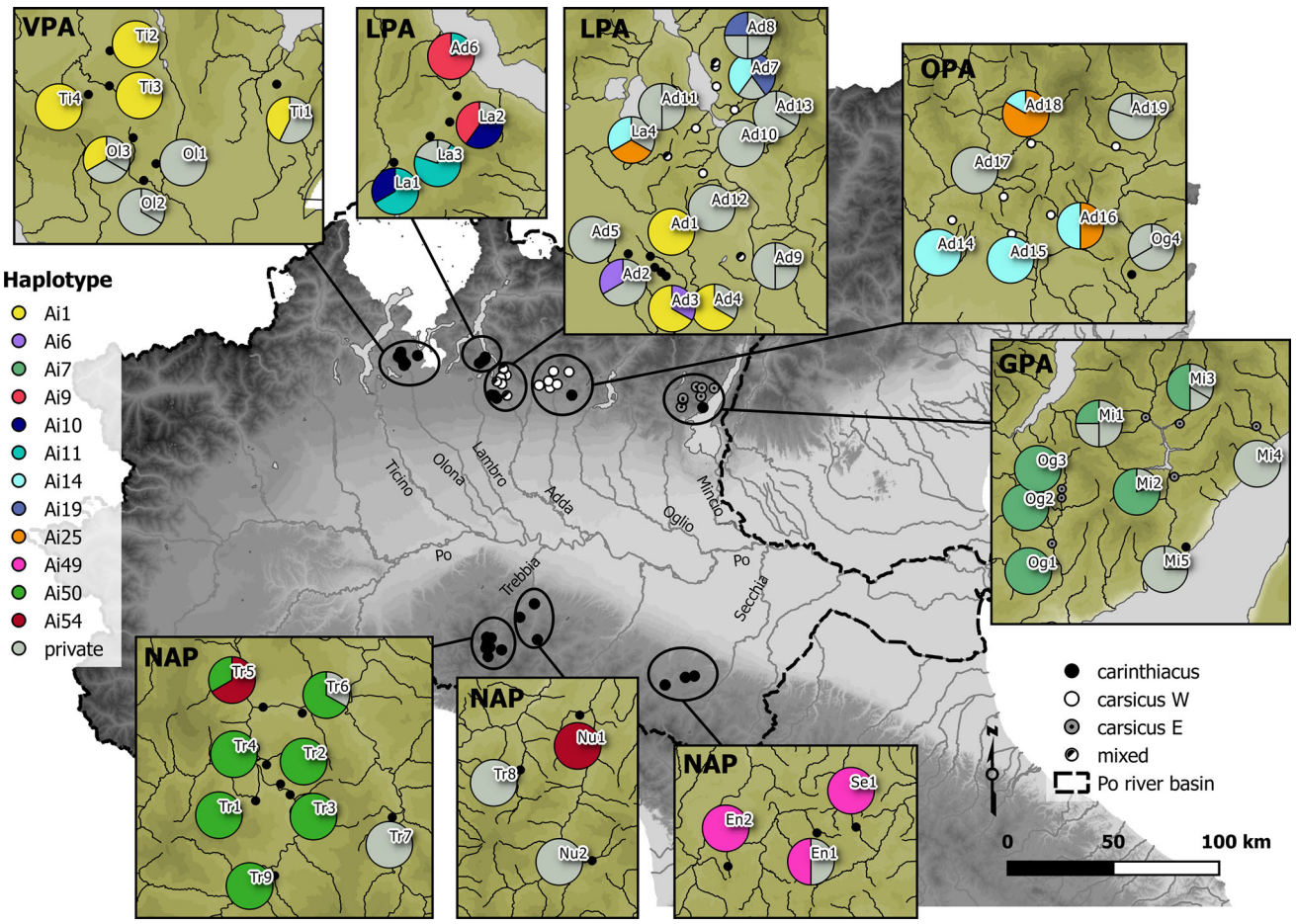

Fig. 1 Sampling localities of Italian white-clawed crayfish populations in Lombardy Prealps (VPA, LPA, OPA, GPA) and in the northern Apennines (NAP). Colours identify alternative clades: *black A. i. carinthiacus*, *white A. i. carsicus W*, *grey circled dot A. i. carsicus E*, *black/white A. i. carinthiacus* and *A. i. carsicus W* mixed populations. *Dotted line* indicates the Po river basin

shaped the present species variability, and the genetic structure of residual populations at local scale.

Thus, DNA aliquots were used to selectively amplify both 3′ and 5′ portions of COI (approximately 1170 base pairs), using two different primer pairs that were specifically designed for this study using the software Primer3 (Rozen and Skaletsky 1998) to target two portions of the *COI* gene: FC_COI5′-F (5′-TTTGGACTTGAGCTGGGATAG-3′) and FC_COI5′-R (5′-AAATTATCCCTAATGTACCAAAAGC-3′); FC_COI3′-F (5′-GCATCTGGATAATCAGAATACC-3′) and FC_COI3′-R (5′-GCATTTGGCATGGTATCACA-3′). Amplifications were conducted in 50 µL reactions containing 1.5 ng of genomic DNA, 10 pmol of each primer, 1X reaction buffer (10 mM Tris–HCl pH9.0, 50 mM KCl, 0.1 % Triton X100), 1.8 mM $MgCl_2$, 0.25 mM of each dNTP, 1 U *Taq* polymerase (Promega Corporation, Madison, WI, USA), 0.5 mg of bovine serum albumine, and ultra-pure H_2O. The thermal cycling profile included a preliminary denaturation cycle of 4 min at 94 °C followed by 32 denaturation-annealing-extension cycles of 30 s at 94 °C, 30 s at 58 °C and 45 s at 72 °C, and a final extension of 7 min at 72 °C. All PCRs were performed in a Bio-Rad thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The obtained amplicons were purified with the PCR clean-up kit Nucleo Spin® Extract II (Macherey-Nagel, Düren, Germany). Sequencing was carried out by Macrogen Laboratories (Amsterdam, The Netherlands), using the same amplification primers.

Phylogenetic analyses

Raw electropherograms were checked by eye using FinchTV (Geospiza Inc., Seattle, WA, USA; available at http://www.geospiza.com), and sequences were aligned using the ClustalW algorithm in BioEdit 7.05 (Hall 1999). The final concatenated alignment (1172 bp) was used to infer phylogenetic relationships with Maximum Likelihood (ML) and Bayesian Inference (BI) approaches, using raxmlGUI 1.3 (Silvestro and Michalak 2012) and MrBayes 3.2 (Ronquist et al. 2012), respectively. The best model of sequence evolution was selected in jModelTest 2.1.2 (Posada 2008), using the corrected Akaike Information Criterion (AICc, Burnham and Anderson 2002). Two homologous sequences of *A. pallipes* from Ligurian Apennines were obtained with the same molecular procedure previously described (*Population sampling and laboratory procedures*) and used as an outgroup in phylogenetic analyses, while homologous sequences from both *A. i. italicus* ($n = 1$, from Tuscany) and *A. i. meridionalis* ($n = 11$, from Abruzzo) were sequenced and included in the phylogenetic analyses for comparison.

A maximum likelihood phylogenetic tree was inferred by performing 100 runs and testing the reliability of the analysis with 1000 bootstrap replicates. Bayesian analysis was performed with a Markov chain Monte Carlo (MCMC) sampling approach. Two independent runs of four chains for each run were carried out for 6×10^6 generations, sampling at intervals of 100th generations. Convergence of chains upon a stationary distribution and appropriate sampling were checked by monitoring the standard deviation of the split frequencies between the two simultaneous runs (<0.001) and the potential scale reduction factor (PSRF) diagnostic (=1.000). Distributions of log-likelihoods and parameter values were examined in TRACER 1.5 (Rambaut and Drummond 2007, available with the BEAST package at http://beast.bio.ed.ac.uk/Tracer) to assess the burn-in threshold, after which MCMC runs converged. After discarding the first 30 % of the trees as burn-in, a majority-rule consensus tree was generated from the remaining trees and visualized in FigTree 1.3.1 (Rambaut 2009). Nodes were considered strongly supported if ML bootstrap proportions were ≥70 % and posterior probability support values ≥0.95 (Wilcox et al. 2002; Huelsenbeck and Rannala 2004). Uncorrected pairwise genetic distances (*p*-distance) between and within clades identified by our phylogenetic analyses were calculated in MEGA 5 (Tamura et al. 2011).

Phylogeographic history and past demographic trends

Mitochondrial gene genealogies among *A. carsicus* and *A. carinthiacus* haplotypes identified were inferred by applying a parsimony approach in order to build haplotype networks, using the software Network 4.6 (Fluxus Technology Ltd; Clare, Suffolk, England; http://www.fluxus-engineering.com). Thus, we followed a median joining procedure (MJ, Bandelt et al. 1999), which yields the best genealogies among other rooting and network procedures (Cassens et al. 2003).

The past demographic history of northern Italian *A. italicus* populations was investigated by performing a mismatch distribution analysis using DNAsp 5 (Librado and Rozas 2009). Specifically, the empirical distributions of nucleotide differences between pairs of sequences ascribed to distinct clades were compared with expectations under a growth-decline model. We assumed that during glacial cycles *A. italicus* populations went through repeated events of contractions into refugia (glacial phases), followed by expansion into formerly glaciated areas (interglacial phases).

Fine-scale population genetic structure

To describe the current spatial distribution of *A. italicus* genetic variation in the investigated area (i.e. Lombardy

Alpine foothills and northern Apennines), we inferred several standard diversity indexes within populations ($n = 53$), hydrographic basins ($n = 10$) and phylogenetic clades, using DNAsp 5 (Librado and Rozas 2009). Specifically, we assessed the haplotype diversity (h, defined as the probability that two haplotypes drawn at random from the population are not the same, Nei 1987), the nucleotide diversity (π, the average number of nucleotide differences per site between two sequences, Nei 1987), and the average number of nucleotide differences (k, Tajima 1983). Populations were further assigned to five groups (Fig. 1) that account for the geographic clustering of samples: the Varese Prealps (VPA), the Larian Prealps (LPA), the Orobian Prealps (OPA), the Gardesan Prealps (GPA), and the Northern Apennine (NAP).

Fine-scale geographic structuring of *A. italicus* populations was investigated by the Analysis of Molecular Variance (AMOVA, Excoffier et al. 1992), looking for the occurrence of a significant hierarchical level of genetic variance. We therefore estimated the Φ-statistic within each phylogenetic clade across either the five spatial groups (VPA, LPA, OPA, GPA and NAP), or different river basins. Statistical support was tested by 10,000 randomized permutations in the software Arlequin 3.5.1.2 (Excoffier et al. 2005). In addition, we performed a number of Mantel tests to investigate the possible pattern of isolation by distance for each pairs of populations. Using the package APE (Paradis et al. 2004) in R ver. 3.1.1 (R Core Team, 2014), we computed correlations between the geographic distances and the genetic distances (pairwise F_{ST}), estimated using Arlequin 3.5.1.2 (Excoffier et al. 2005) with 1000 permutations. The geographic distances (km) were retrieved from the Po basin hydrography using the shortest waterways between sampling sites in GIS environment (QGIS 2.6.1). Mantel tests were performed only with sample size of $n > 4$. In this way, we evaluated the extent of isolation of populations at different levels, from geographic area to basin scale, in a conservation perspective.

Results

Pattern of sequence variation

All 205 *A. italicus* samples and 14 outgroup samples were successfully processed and returned a final alignment of 1172 bp. Considering the 205 sequences obtained from our study area, 125 sites were polymorphic, and 109 parsimony informative (87.2 %). When the outgroup samples were included, 192 variable sites were observed, with 81.3 % parsimony informative. The average base pair sequence composition was T = 38.7 %, C = 15.2 %, A = 24.7 % and G = 21.4 %. Overall 56 haplotypes were found from our study area (all the 56 haplotypes were deposited in GenBank under accession numbers from KR232582 to KR232637). Out of the 56 *COI* haplotypes identified in the Po river basin, 33 belonged to *A. i. carinthiacus* ($n = 125$) and 23 to *A. i. carsicus* ($n = 80$) (see next section for phylogenetic assignment of haplotypes). Only 12 haplotypes were shared by at least two populations (see Online Resources 2 and Fig. 1), while most of them were private ($n = 44$; 78.6 %), i.e. they occurred in a single population. The most widespread haplotypes were Ai50 and Ai1 (assigned to *A. i. carinthiacus* by our phylogenetic analysis, see next section), shared respectively by 12.7 % (7 populations, $n = 26$) and 11.2 % (8 populations, $n = 23$) of the samples, while Ai7 was shared by 12.2 % (6 populations, $n = 25$) of *A. i. carsicus* (see Fig. 1).

Phylogenetic analyses

According to the AICc, the best substitution model selected for our dataset was GTR+I+G. Both ML and BI tree topologies were highly comparable and revealed the presence of deeply differentiated mitochondrial clades within *A. italicus* (Fig. 2). In particular, *A. i. meridionalis* (ML = 100, BI = 1.00) was well differentiated from *A. i. carsicus* (ML = 94, BI = 0.92), *A. i. carinthiacus* (ML = 83, BI $\leq$ 0.95), and the only sequence of *A. i. italicus* considered in our analyses. Though the latter was definitely differentiated from *A. i. carinthiacus*, the two subspecies appear to be closely related to each other within *A. italicus* (Fig. 2). Interestingly, two distinct and divergent sister clades occurred within *A. i. carsicus*. We tentatively named the populations sampled in the central Lombardy (ML = 99, BI = 1.00) as the "western (*W*) clade", and the populations from the eastern Lombardy (ML = 98, BI = 1.00) as the "eastern (*E*) clade", hereafter referred as *carsicus W* and *carsicus E* respectively.

Uncorrected pairwise (p) genetic distances between main phylogenetic clades and subspecies were lower than within them (Table 1). The highest differentiation (6.06 %) occurred between *A. i carsicus W* and *A. i. meridionalis*, while the lowest (1.80 %) was recovered between *A. i. carinthiacus* and *A. i. italicus*. Molecular differentiation between the two clades of *A. i. carsicus* (*W* and *E*) was 3.03 %. Higher genetic differentiation values were observed between each clade and *A. pallipes* (see Table 1 for all comparison values).

Phylogeographic and past demographic trends

The most parsimonious MJ network ($\varepsilon = 0$, Fig. 3) showed three distinct clusters in which 56 haplotypes were joined with the addition of 12 median vectors. A single network

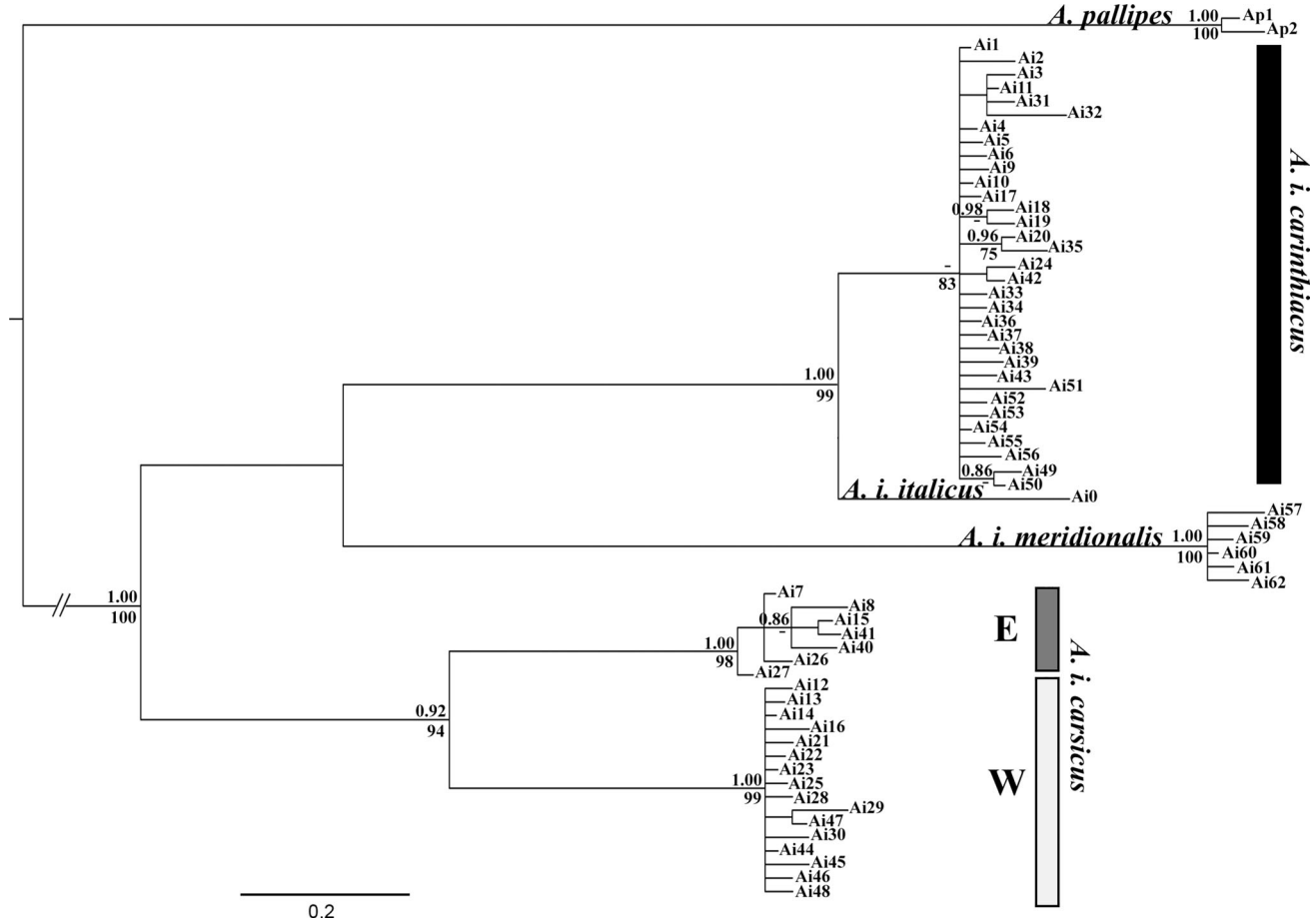

Fig. 2 Phylogenetic tree showing relationships within *A. italicus*. The best substitution model of evolution was GTR+I+G according to the corrected Akaike Information Criterion (AICc). Maximum Likelihood bootstrap supports (below nodes, 75 % threshold) were estimated by performing 100 runs with 1000 bootstrap replicates. Bayesian posterior probabilities (above nodes, 0.85 threshold) were estimated with Markov chain Monte Carlo (MCMC) sampling approach, running two runs and four chains for each run for 6 million generations and sampling every 100th tree. The first 30 % of the data were discarded as burn-in after checking for runs convergence in TRACER 1

Table 1 Uncorrected pairwise (*p*) genetic distances between (*below the diagonal*) and within (*along the diagonal*, in italics) phylogenetic clades identified in this study

Clades		[1]	[2]	[3]	[4]	[5]	[6]
A. i. carsicus E	[1]	*0.0028 ± 0.0009*					
A. i. carsicus W	[2]	0.0303 ± 0.0049	*0.0026 ± 0.0007*				
A. i. carinthiacus	[3]	0.0547 ± 0.0062	0.0514 ± 0.0061	*0.0034 ± 0.0006*			
A. i. italicus	[4]	0.0527 ± 0.0060	0.0520 ± 0.0062	0.0180 ± 0.0036	*n/c*		
A. i. meridionalis	[5]	0.0536 ± 0.0064	0.0606 ± 0.0067	0.0580 ± 0.0064	0.0588 ± 0.0068	*0.0031 ± 0.0009*	
A. pallipes	[6]	0.0709 ± 0.0074	0.0709 ± 0.0078	0.0700 ± 0.0069	0.0742 ± 0.0073	0.0802 ± 0.0077	*0.0026 ± 0.0015*

Standard errors (±) are also given

joined together all the haplotypes of *A. i. carinthiacus* from both Alpine and Apennines streams, while the two *A. i. carsicus* clades (*W* and *E*), differed by at least 31 nucleotide substitutions. In the *A. i. carinthiacus* network, connections among several haplotypes remained unresolved (i.e. more than one parsimonious solution to connect them were proposed), suggesting the occurrence of several extinct or unsampled haplotypes in our dataset for this

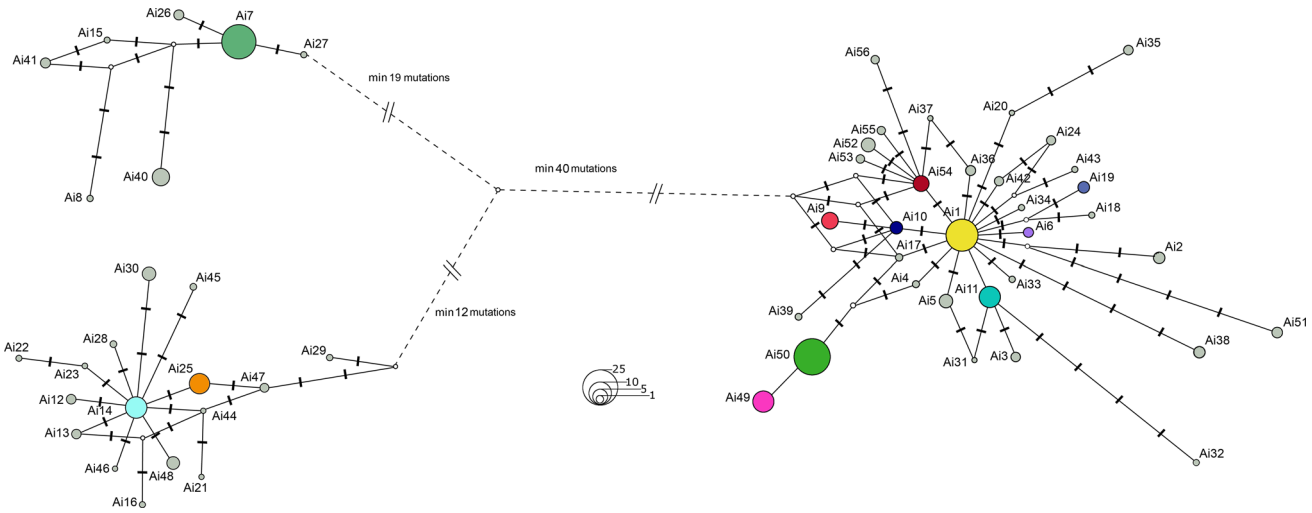

Fig. 3 Most parsimonious (ε = 0) MJ network of 205 COI mtDNA sequences of *A. italicus*. Haplotype circles dimension is proportional to haplotype frequency. Haplotypes are identified by the different colours used in Fig. 1; all private haplotypes are grey coloured. White circles without haplotype code represent median vectors indicating hypothetical missing or ancestral not sampled haplotypes. Each tick-mark on solid lines connecting haplotypes represents single mutation between them. Dotted lines indicate minimum number of substitutions required to connect networks

subspecies. *A. i. carsicus W* showed a typical star-like pattern compatible with a recent expansion of this clade in the surveyed area, while *A. i. carsicus E* appeared more structured, despite the lower number of haplotypes sampled.

Reconstruction of the mismatch distribution profile for *A. i. carsicus* clearly showed two sharp peaks, further supporting the occurrence of two deeply differentiated evolutionary lineages within this subspecies (Fig. 4a). The ragged profile observed for clade *E* was compatible with a scenario of repeated contractions followed by new expansion (Fig. 4b), whereas the unimodal profile detected for clade *W* matched the one resulting from a single recolonization event (Fig. 4c). No similar structure emerged within *A. i. carinthiacus* (Fig. 4d), which again showed a profile consistent with a single recolonization event.

Fine-scale population genetic structure

In the surveyed area, populations of the two subspecies showed mainly parapatric distribution patterns, with limited overlap in the Adda and Lambro river basins (Fig. 1). In VPA (Ticino and Olona river basins) only *A. i. carinthiacus* was detected. Moving eastward, mixed populations of *A. i. carinthiacus* and *A. i. carsicus* were found in four streams in LPA, one located in the Lambro river basin (locality La4) and three in the Adda river basin (localities Ad7, Ad8, Ad9). However, the majority (57.1 %) of the populations sampled in the Adda river basin held *A. i. carsicus W* haplotypes. In the OPA and GPA, most populations were composed of *A. i. carsicus*, particularly by members of the *W* clade in OPA (Adda river basin) and the *E* clade in GPA (Oglio and Mincio river basins). Only two populations ascribed to *A. i. carinthiacus* (localities Og4 and Mi5, respectively) occurred in these areas (Fig. 1). Thus, we excluded them from subsequent population analyses following precautionary principle. Finally, only *A. i. carinthiacus* occurred in the four Apennine river basins (Trebbia, Nure, Enza and Secchia, NAP).

Overall, haplotype diversity (h) and nucleotide diversity (π) within our dataset (excluding the outgroups; Table 2) were high (0.946 ± 0.007 and 0.02840 ± 0.00113, respectively), with an average of 33.28 nucleotide differences (k). Considering the investigated area, *A. i. carinthiacus* returned the highest intra-clade genetic diversity ($h = 0.909 \pm 0.015$, $\pi = 0.00275 \pm 0.00016$ and $k = 3.22$). Within *A. i. carsicus*, all diversity values, particularly haplotype diversity, were higher within clade *W* ($h = 0.880 \pm 0.032$, $\pi = 0.00166 \pm 0.00021$ and $k = 1.95$) than *E* ($h = 0.549 \pm 0.088$, $\pi = 0.00128 \pm 0.00025$ and $k = 1.50$).

In VPA (only *A. i. carinthiacus*), high diversity values were recovered within the Olona basin, where several private haplotypes were found (Table 2). LPA returned the highest diversity values as a result of the presence of both *A. i. carinthiacus* and *A. i. carsicus W* populations (particularly in the Adda river basin), with the latter showing only private haplotypes (Table 2). In this area of "mixed" populations, nucleotide diversity (π) was particularly high, ranging from 0.0246 to 0.03356 versus 0 to 0.00512 in all other populations. Similarly, the highest average number of nucleotide differences (k) was observed in the mixed population La4 from the Lambro river basin (=39.33, see

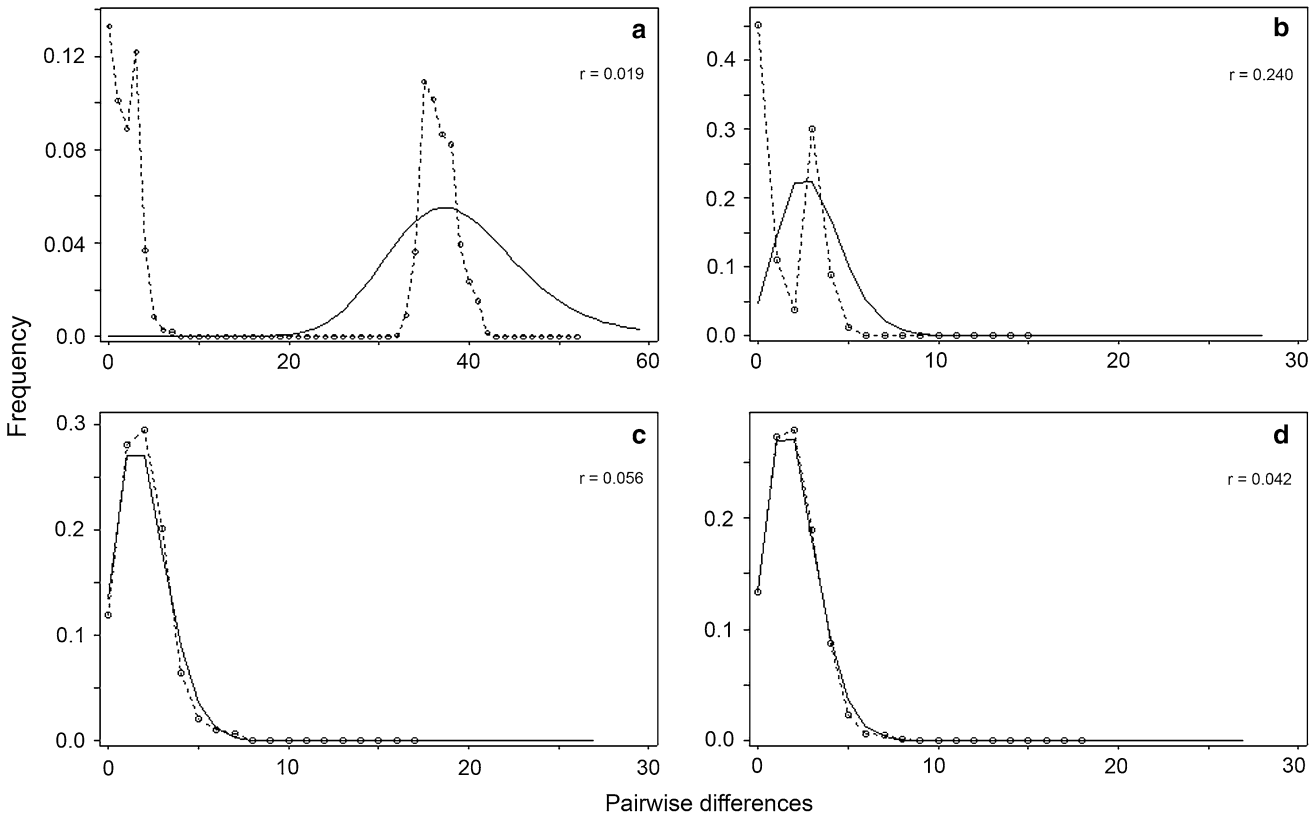

Fig. 4 Mismatch distribution profiles (*dotted lines*): total *A. i. carsicus*, **a** *A. i. carsicus* clade *E*, **b** *A. i. carsicus* clade *W*, **c** *A. i. carinthiacus*, **d** simulated plots assuming growth-decline model of population growth are also shown (*solid lines*). *r* raggedness index

Online Resource 3). In OPA (only *A. i. carsicus W*) populations appeared overall more variable than in GPA (only *A. i. carsicus E*), where several monomorphic populations were found to share a single haplotype (particularly in the Oglio basin). Where haplotype Ai7 also inhabited the Mincio basin, variation was high compared to the Oglio basin (Table 2) Finally, considering only *A. i. carinthiacus*, Apennine populations (NAP) appeared overall less variable than Alpine ones, with only three polymorphic populations occurring in two basins (Trebbia and Enza), each with just two different haplotypes (Table 2).

The analysis of molecular variance (AMOVA) revealed significant genetic structuring of molecular variation within our dataset at different hierarchical levels of investigation (geographic areas, river basins and populations) (Table 3). Considering each clade separately (except introduced or "mixed" populations), clear genetic structuring was recovered within *A. i. carinthiacus*, for which a significant proportion of the observed variance was explained by assigning populations to different geographic areas (VPA vs. LPA vs. NAP, 34.99 %,) or different river basins (38.87 %). By contrast, no significant structuring was observed among basins within the same geographic areas (not shown). Anyway, the occurrence of two spatially disconnected population groups (the Alpine group vs. the Apennine group, see Fig. 1) explained the highest percentage of variation within this clade (41.18 %) (Table 3). Instead, within each *A. i. carsicus* clade no significant structure was observed among groups (Table 3). Most of the genetic variation within each *A. i carsicus* clade was found among populations within groups or within populations (40.40 and 52.15 % respectively; significant components).

The Mantel tests performed within each clade did not detect significant correlation for *A. i. carinthiacus* ($r = 0.27$, $p = 0.073$), or the two clades *A. i. carsicus W* ($r = 0.04$, $p = 0.27$) and *A. i. carsicus E* ($r = 0.24$, $p = 0.27$). The Mantel tests performed for populations within geographic area did not detect any significant correlation for the GPA, OPA, VPA and NAP areas (Table 4), but did for the Trebbia river basin ($r = 0.70$, $p = 0.011$).

Discussion

Cryptic genetic variation within northern Italian populations of *A. italicus*

Our phylogenetic analysis of the residual populations of *A. italicus* from the Lombardy Alpine foothills and northern Apennines river basins provides new and important

Table 2 Genetic diversity indexes recovered for the whole dataset, each clade, geographic areas, river basins: (n) number of samples, (n_H) number of haplotypes, (*Clade*) clade recognized by our phylogenetic inference, (k) average number of nucleotide differences, (h) haplotype diversity, (π) nucleotide diversity, (Sd) standard deviation

	n	n_H		k	h (Sd)	π (Sd)
Overall populations	205	56		33.28	0.946 (0.007)	0.02840 (0.00113)
A. i. carinthiacus	125	33		3.22	0.909 (0.015)	0.00275 (0.00016)
A. i. carsicus W	42	16		1.95	0.880 (0.032)	0.00166 (0.00021)
A. i. carsicus E	38	7		1.50	0.549 (0.088)	0.00128 (0.00025)
Area	n	n_H	*Clade*	k	h (Sd)	π (Sd)
VPA (Ti1–Ti4; Ol1–Ol3)	24	7	*A. i. carinthiacus*	0.93	0.688 (0.093)	0.00079 (0.00016)
LPA	57	28	*A. i. carinthiacus,*	26.35	0.940 (0.015)	0.02248 (0.01403)
(La1–La4; Ad1–Ad13)			*A. i. carsicus W*			
OPA (Ad14–Ad19)	22	5	*A. i. carsicus W*	1.58	0.732 (0.054)	0.00135 (0.00032)
GPA (Og1–Og3; Mi1–Mi4)	38	7	*A. i. carsicus E*	1.50	0.549 (0.088)	0.00128 (0.00183)
NAP (Tr1–Tr9; Nu1–Nu2; En1–En2; Se1)	47	8	*A. i. carinthiacus*	2.53	0.657 (0.066)	0.00216 (0.00038)
River basin	n	n_H	*Clade*	k	h (Sd)	π (Sd)
Ticino (Ti)	16	2	*A. i. carinthiacus*	0.40	0.400 (0.114)	0.00034 (0.00010)
Olona (Ol)	8	6	*A. i. carinthiacus*	1.71	0.929 (0.084)	0.00146 (0.00023)
Lambro (La)	21	9	*A. i. carinthiacus, A. i. carsicus W*	12.02	0.805 (0.079)	0.01026 (0.00494)
Adda (Ad)	68	26	*A. i. carinthiacus, A. i. carsicus W*	29.45	0.937 (0.014)	0.02513 (0.00113)
Oglio (Og)	17	1	*A. i. carsicus E*	–	–	–
Mincio (Mi)	21	7	*A. i. carsicus E*	2.36	0.786 (0.063)	0.00202 (0.00026)
Trebbia (Tr)	33	5	*A. i. carinthiacus*	1.67	0.377 (0.103)	0.00143 (0.00036)
Nure (Nu)	3	2	*A. i. carinthiacus*	1.33	0.667 (0.314)	0.00114 (0.00054)
Enza (En)	7	2	*A. i. carinthiacus*	4.29	0.476 (0.171)	0.00366 (0.00132)
Secchia (Se)	4	1	*A. i. carinthiacus*	–	–	–

Populations for which human-mediated introduction is suspected have been excluded

insights into the complex phylogenetic framework and genetic variation of this endangered freshwater crayfish. We precisely delineated the distribution of the two distinct evolutionary significant units (ESUs), deserving subspecific status (*A. i carinthiacus* and *A. i. carsicus*) already detected in northern Italy (e.g. Fratini et al. 2005). Moreover, we highlight the presence of two well-differentiated lineages, *E* and *W* within *A. i. carsicus*, in the OPA and GPA areas respectively (Fig. 1). No mixed populations and no overlapping areas were identified between the two *carsicus* lineages. Our phylogenetic results are consistent with the median-joining parsimony network analysis, in which clades *W* and *E* appear disconnected by several mutations. Moreover, the observed genetic distance between *W* and *E* lineages was only slightly lower than those recorded between each *carsicus* lineage and *A. i. carinthiacus*, while the genetic variation within them was mostly similar. However, it cannot be ruled out that this distinction within *A. i. carsicus* could be due to sub-optimal sampling in the area between OPA and GPA. Further research in this area is required to locate new white-clawed crayfish populations and to investigate their genetic structure. Furthermore no comparable diversity was recorded within *A. i. carinthiacus*, while geographically distant (in the Apennines and the Alps) populations were sampled.

Biogeographic considerations

According to our results, the cryptic genetic differentiation observed within *A. carsicus* should be the result of past disconnections and multiple extinctions/recolonizations by the Alpine populations during and after Pleistocene glaciations. Ice-ages have been widely recognized as major factors shaping the biogeography of the endemic species in northern Italy (Hewitt 1996; Taberlet et al. 1998; Hewitt 2000). Interestingly, both phylogeographic and population

Table 3 Summary of the AMOVA performed on phylogenetic clades and the specified groups recovered in our study area (river basins and geographic areas)

Source of variation	d.f.	SS	Variance components	%	*p*	Φ-statistic
***A. i. carinthiacus* (Ti, Ol, Ad, La, Tr, Nu, En, Se)**						
Among groups	7	74.10	0.61	38.87	<0.001	$\Phi_{CT} = 0.389$
Among populations within group	22	52.44	0.55	34.61	<0.001	$\Phi_{SC} = 0.566$
Within populations	82	34.28	0.42	26.52	<0.001	$\Phi_{ST} = 0.735$
Total	111	160.82	1.58			
***A. i. carinthiacus* (VPA, LPA, NAP)**						
Among groups	2	49.25	0.59	34.99	<0.001	$\Phi_{CT} = 0.350$
Among populations within group	27	77.29	0.67	40.07	<0.001	$\Phi_{SC} = 0.616$
Within populations	82	34.28	0.42	24.94	<0.001	$\Phi_{ST} = 0.751$
Total	111	160.82	1.68			
***A. i. carinthiacus* (Alps vs. Apennine)**						
Among groups	1	45.24	0.77	41.18	<0.001	$\Phi_{CT} = 0.412$
Among populations within group	28	81.31	0.68	36.35	<0.001	$\Phi_{SC} = 0.618$
Within populations	82	34.28	0.42	22.47	<0.001	$\Phi_{ST} = 0.776$
Total	111	160.82	1.86			
***A. i. carsicus E* (Mincio vs. Oglio)**						
Among groups	1	2.30	0.07	21.39	n.s.	$\Phi_{CT} = 0.214$
Among populations within group	5	3.97	0.13	40.40	<0.001	$\Phi_{SC} = 0.514$
Within populations	31	3.88	0.12	38.20	<0.001	$\Phi_{ST} = 0.618$
Total	37	10.16	0.33			
***A. i. carsicus W* (LPA vs. OPA; Adda)**						
Among groups	1	2.37	0.01	1.43	n.s.	$\Phi_{CT} = 0.949$
Among populations within group	8	16.47	0.49	46.42	<0.001	$\Phi_{SC} = 0.568$
Within populations	22	12.10	0.55	52.15	<0.001	$\Phi_{ST} = 0.978$
Total	31	30.91	1.05			

Mixed or introduced populations have been not considered

genetics analyses support this conclusion: *A. i. carsicus* haplotypes fell into two deeply divergent networks (Fig. 3) and underwent alternative demographic trends in the past (Fig. 4). Similarly genetic variation among OPA and GPA match the occurrence of distinct phylogenetic clades (*W* and *E*) in these areas (Table 3). We therefore hypothesize that these two separate lineages probably survived glaciations in at least two distinct glacial refugia, respectively located in western and eastern Alps. The mismatch analysis performed on *A. i. carsicus* as a whole similarly showed a clear bimodal frequency distribution of pairwise nucleotide differences among sequences. This pattern usually suggests the occurrence of undetected and deeply differentiated ancient evolutionary lineages within the investigated taxon. The ragged profile observed for clade *E* suggests that the populations have experienced repeated contractions followed by new expansions, a pattern consistent with the demographic scenarios expected in glacial refugia (Fig. 4b). By contrast, the unimodal profile detected for clade *W* is compatible with a single recolonization event by populations from a glacial refugium located elsewhere (Fig. 4c).

Although previous works suggested the occurrence of an eastern glacial refugium of *A. italicus* in the Istrian peninsula (Trontelj et al. 2005), our results support the hypothesis that *carsicus* populations could have survived glaciations in more than one Alpine refugium, and particularly in the area of the Italian peninsula where we sampled populations ascribed to clade *E* (specifically in the Mincio river basin). In contrast, populations ascribed to clade *W* could have persisted glaciations in a refugium either located in the southern Po plain, which extended into the now Adriatic basin, or in multiple microrefugia located in the southern slopes of the OPA area. The presence of multiple glacial refugia south of the Alps had already been proposed by several studies, highlighting a common pattern for different Alpine animal and plant species (Scotti et al. 2000; Stehlik 2000; Schönswetter et al. 2005; Stefani et al. 2012). In *A. i. carinthiacus*, the mismatch profile suggests a rapid recolonization of the northern Apennines and central

Table 4 Summary of Mantel tests comparing effects of geographic distances (waterways) into genetic divergence estimated by pairwise F_{ST}. (r) Mantel's correlation

	r	*p*
Clades		
A. i. carinthiacus	0.27	0.073
A. i. carsicus W	0.04	0.270
A. i. carsicus E	0.24	0.270
Geographical areas		
GPA	0.24	0.270
OPA	0.06	0.180
LPA	n.c.	n.c.
VPA	0.48	0.079
NAP	0.39	0.053
River basin		
Trebbia	0.70	0.011
Adda	n.c.	n.c.

Mixture of clades or introduced populations have been not considered (n.c.)

Alps after the last Pleistocene glaciations, but no evidence for multiple glacial refugia was identified for the clade in our study area.

Contact zone

One of the most important results of our study is the clear definition of a contact zone between *carinthiacus* and *carsicus* lineages in the Adda and Lambro river valleys. In particular, haplotypes of both these subspecies occur in syntopy, at least in the four populations we sampled in the Adda (localities Ad7, Ad8, Ad9) and Lambro (locality La4) river basins. The detection of these mixed populations indicates that the two subspecies overlap in Larian Prealps but not in Orobian Prealps, as formerly hypothesized (Fratini et al. 2005). Thus, the borderline distribution of these two ESUs is accurately delimited by our results, with mixed populations detected also along the watershed between Lambro and Adda river basins (see LPA in Fig. 1). The presence of populations in which *carsicus* and *carinthiacus* haplotypes occur in syntopy could be the outcome of human translocations, a practice documented for the past and likely to have affected the white-clawed crayfish distribution all over Europe (Souty-Grosset et al. 1997; Gouin et al. 2003; Stefani et al. 2011). Nevertheless, we believe that the observed scenario is rather the result of secondary contacts between two deeply differentiated clades after their last post-glacial expansion. Indeed, in mixed populations the occurrence of at least seven private haplotypes of both lineages could hardly be explained by founder effects due to human translocation. In contrast, populations Og4 and Mi5 (in the OPA and GPA areas, respectively), showing *carinthiacus* mtDNA, but occurring within the geographic range of *A. i. carsicus,* may derive from past human translocations. Although the haplotypes are private, two very different haplotypes (Ai38 and Ai39, differing at least by seven mutations each other, see Fig. 3) recovered in population Mi5 suggest a low chance that this is a relict population.

Remarks for conservation and management

Our results indicate the existence of two differentiated and previously undetected phylogenetic lineages within *A. i. carsicus*, with alternative geographic distribution and with different patterns of genetic variation. We suggest that these two units should be considered as new molecular operational taxonomic units (MOTUs). In the Adda river basin the co-occurrence of two MOTUs has to be carefully considered and crayfish should not be stocked from the mixed populations. The AMOVA test showed for *A.i. carinthiacus* that the genetic component associated with the geographic areas or river basins was significant, but also population structure within rivers or within geographic areas is actually quite high. Indeed, for each *A. i. carsicus* clade most genetic variance could be explained by variation within populations or within rivers. Moreover, the Mantel test did not detect significant isolation-by-distance pattern within GPA, OPA, VPA and NAP. These results suggest that populations have to be managed using close attention. After genetically mapping each stream, only translocations among populations genetically close could be done. Moreover in the Trebbia river basin, only translocations among geographically near populations are approved, due to the high correlation detected by Mantel test. We suggest that all these remarks deserve special attention in future conservation and management plans. Indeed, any conservation strategy for the white-clawed crayfish should aim to not only increase the number of individuals, but also to promote the maintenance of genetically distinct lineages, each representing an important biodiversity reservoir (Bertocchi et al. 2008).

Concerning the contact zone between *A. i carsicus* and *A. i. carinthiacus* in the LPA, further study involving co-dominant and more sensitive markers, such as nuclear microsatellites, would be highly desirable in order to clarify the occurrence of effective gene flow and identify distinct reproductive groups, particularly in the areas of syntopy. Indeed, management plans involving selective processes promoting speciation (like, background selection of the hybrids) could not be ignored in order to ensure a successful restocking of populations, to avoid polluting the gene pools, and to ultimately avoid money waste.

Overall, our fine-scale sampling approach of the residual northern Italian populations of this endangered crayfish allowed us to detect an extreme level of genetic variation despite the relatively small geographic area investigated. Our results of nucleotide diversity are in accordance with Trontelj et al. (2005) for a wider geographic area (NW Italy + S Switzerland), while our levels of genetic variability show higher values than those previously found in the Po river basin (Zaccara et al. 2005). The high occurrence of private haplotypes, already found in previous studies (Trontelj et al. 2005; Zaccara et al. 2005), and the high proportion of localities with at least one private haplotype, further suggests strong isolation and a low gene flow among populations inhabiting not only different river basins, but also different tributaries of the same basin.

Our findings suggest that any conservation strategy for *A. italicus* in northern Italy must take into account both the complexity of the biogeographic pattern, and the progressive isolation of local demes. Indeed, conservation efforts should be aimed at restoring/increasing gene flow between residual populations belonging to the same subspecies or MOTU, according to the geographic and genetic distance of the populations, thus ensuring that any reproductive barrier would not incur if reproductive crayfish were translocated (Storfer 1999; Edmands 2002; Galeotti et al. 2012).

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