Genetic characterization of *Austropotamobius pallipes* (Lereboullet, 1858) *complex* in Friuli Venezia Giulia for restocking purposes

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Abstract

The white-clawed crayfish *A. pallipes* has suffered in recent decades a strong decline throughout its entire distributional range, mainly due to the growing number of threats coming from anthropic influence, including habitat loss and degradation, overfishing, infectious diseases, and the introduction of non-indigenous crayfish species (NICS). The species is included in the red list of the IUCN (International Union for Conservation of Nature) as a species at risk of extinction. An important goal in conservation biology is to assess the genetic variability and thus the “genetic health” of populations and to identify any evolutionarily significant unit (ESU) within endangered species, before management decisions are taken.

Within RARITY (http://www.life-rarity.eu), a LIFE project for the eradication of the invasive Louisiana red swamp (*Procambarus clarkii*) and for the preservation of the native white-clawed crayfish in Friuli Venezia Giulia (NE Italy), I was responsible for the genetic characterization of *A. pallipes complex* in this area, in order to define the taxonomic status, the genetic variability and the population structure and differentiation. The analysis of two mitochondrial genes (COI and 16 rDNA) of about 500 individuals from 58 monitored sites showed that the FVG crayfish belong to the *A. italicus* species, with two different subspecies present: *A.i. carsicus* and *A.i. meridionalis*. The analysis at six polymorphic microsatellite loci revealed generally low levels of within population genetic diversity (0.0 <Ho< 0.5) with overall high inbreeding coefficients (average F=0.422), likely as a result of genetic drift in small sized populations. FVG populations appeared significantly differentiated among the different river drainages and were highly structured within rivers displaying a significant pattern of isolation by distance, suggesting significant habitat fragmentation of steam ecosystems.

The combination of mitochondrial and microsatellite markers allowed us to identify two ESUs, corresponding to the two subspecies in northern and southern FVG (Rosandra stream). Our data also support the maintenance of separate of separate demographic management strategies for crayfish inhabiting different drainage systems. This study provided new knowledge on white-clawed crayfish populations to create “genetic maps” that can be used as valuable data for restocking practices and conservation programs in FVG.
Riassunto

Le popolazioni del gambero di fiume *A. pallipes* hanno subito negli ultimi decenni un forte declino lungo l'intero areale di distribuzione, principalmente a causa del crescente numero di minacce attribuibili alla crescente influenza antropica, tra cui la riduzione e il degrado dell'habitat, la pesca eccessiva, le malattie infettive, e l'introduzione di specie di gambero non indigene (NICS). La specie è inclusa nella lista rossa della IUCN (Unione Internazionale per la Conservazione della Natura) come specie a rischio di estinzione. Un importante obiettivo della biologia della conservazione è quello di valutare la variabilità genetica come stima indiretta dello "stato di salute" delle popolazioni e di identificare popolazioni altamente differenziate, definite Unità Evolutivamente Significative (ESU), all'interno delle specie a rischio di estinzione, prima di adottare decisioni gestionali.

All'interno di RARITY (http://www.life-rarity.eu), un progetto LIFE per l'eradicazione della specie invasiva del gambero rosso della Louisiana (*Procambarus clarkii*) e per la conservazione del gambero di fiume nativo in Friuli Venezia Giulia (NE Italia), sono stata responsabile della caratterizzazione genetica del complesso di specie *A. pallipes* in regione, per definire lo status tassonomico, valutare la variabilità genetica e il differenziamento delle popolazioni gambericole. L'analisi di due regioni mitocondriali (COI e 16 rDNA) di circa 500 esemplari provenienti da 58 siti di monitoraggio ha dimostrato che i gamberi del FVG appartengono alla specie *A. italicus*, con due sottospecie diverse presenti: *A.i. carsicus* e *A.i. meridionalis*. L'analisi di sei loci microsatelliti polimorfici ha rivelato livelli generalmente bassi di variabilità genetica all'interno delle popolazioni (0,0 <Ho <0,5), con coefficienti di inincrocio complessivamente elevati e significativi (F = 0,422), probabilmente a causa dell'effetto della deriva genetica in popolazioni di piccole dimensioni. Inoltre è emerso che le popolazioni del FVG sono notevolmente differenziate e strutturate geneticamente sia tra i diversi bacini idrici che all'interno degli stessi, seguendo un modello di isolamento da distanza, che suggerisce una significativa frammentazione degli ecosistemi fluviali regionali.

L'uso combinato di marcatori mitocondriali e nucleari ha permesso di identificare due ESU, corrispondenti alle due sottospecie presenti rispettivamente in FVG settentrionale e meridionale (Val Rosandra). I dati ottenuti supportano inoltre il mantenimento di strategie di gestione demografica separate per popolazioni appartenenti ai diversi bacini idrici. Questo studio ha fornito nuove conoscenze sul gambero di fiume autoctono in FVG, creando una “mappatura genetica” delle popolazioni che può essere utilizzata come valido supporto informativo di futuri programmi di conservazione, specialmente legati al ripopolamento e/o alla reintroduzione della specie.
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Bibliography
1. Introduction

1.1. Conservation status of *A. pallipes* complex and the Rarity project

The distribution range of indigenous crayfish species (ICS) in Europe has drastically decreased in the last fifty years, mainly due to the growing number of threats coming from anthropic influence, including habitat loss and degradation, overfishing, infectious diseases, and the introduction of non-indigenous crayfish species (NICS) (Gherardi et al., 1999); (Holdich, D.M. et al., 2009). Specifically, this decline has interested the white-clawed crayfish *Austropotamobius pallipes*, that is today classified as endangered by the IUCN (Fürerder et al., 2010) whereas it was only classed as vulnerable in 1996 (Baillie, 1996). In the last ten years this species is suspected to have undergone a decline of somewhere between 50–80% based on presence/absence data available for England, France and Italy (Fürerder et al., 2010).

Due to their important role in freshwater communities, declines in this keystone species are thought to negatively impact both ecosystem structure and function within freshwater environments (Holdich et al., 2002). Today the species is listed under the EU Habitats Directive Annex II and V and therefore requires the designation of special areas of conservation for its protection.

As in the rest of Europe, crayfish populations have declined drastically all over Italy due to the multiplication of the threats to their integrity mainly coming from the increasing human pressures. Although the white-clawed crayfish may still be abundant in some restricted areas of its range, population densities have declined enormously in the past few years and its range has been drastically reduced (Kozák et al., 2011; Mazza et al., 2011). In northern Italy, populations of this species (Bertocchi et al., 2008; Fratini et al., 2005; Scalici and Bravi, 2012; Zaccara et al., 2004) are becoming more and more rare, and in several territories are at the edge of extinction (Brusconi et al., 2008). South Tyrol is thought to be exhibiting some of the greatest declines in the abundance of this species. The introduction of *Pacifastacus leniusculus* in 1981 from Austria in the South Tyrol region of Italy, may have led to the disappearance of *A. pallipes* in that area (Fürerder and Machino, 1999). A significant decline in the number of populations within Liguria, Piedmont, and Tuscany has also been observed (Brusconi et al., 2008; Souty-Grosset et al., 2006).

The Habitats directive was transposed in Italy through Presidential Decree 357/1997 instituting the Natura 2000 network and, which places strong restrictions on the introduction into the wild of non-local species. The decree requires regional authorities to adopt all possible measures for
maintaining in a satisfactory state of conservation the species of EU interest and monitoring of the relative populations. The great concern for the conservation status of the species in Italy has led, over the past few years, various attempts to protect ICS and to re-establish extinct populations through the development of two main and complementary objectives of crayfish management: (1) the conservation of ICS populations and their genetic diversity and (2) control or eradication of invasive populations of NICS. Some projects aimed at managing ICS have been co-funded by the European Union through the programme LIFE (“L’Instrument Financier pour l’Environnement”). Most recent projects for Conservation and recovery of A. pallipes were carried out in northern Italy (Lombardy) and central Italy (Abruzzo Region).

Moreover, the conservation status of white clawed crayfish is further complicated by a controversial taxonomic status. Genetic studies showed that A. pallipes is a species complex with a pronounced genetic structure at both the inter and intra – specific level. (Grandjean et al., 2002, 2000). The complex consists of two genetically distinct species: A. pallipes e A. italicus. in the Italian territory A. pallipes is confined to the north-western regions, while A. italicus is distributed in the rest of continental and peninsular Italy. The situation is even more complicated considering that A. italicus has four subspecies: A. i. italicus in the Tuscan-Emilian Apennines, A. i. carsicus in the north-eastern regions, A. i. carinthiacus in the central and north-western regions and A. i. meridionalis in the central and southern regions (Fratini et al., 2005).

European law protects A. pallipes which in fact corresponds locally to several threatened species/subspecies not named in the legislation. Since in a correct species conservation policy subspecific entities should be regarded as distinct "management units", any protection plan involving reintroduction/restocking should be preceded by the genetic characterization of the populations involved.

Friuli Venezia Giulia region (NE Italy) represents a hot spot for crayfish diversity since it is the only Italian region to harbour, besides the white-clawed crayfish, the two other Italian ICS, which are the noble crayfish, Astacus astacus, and the stone crayfish, Austropotamobius torrentium. Up to now, populations of A. torrentium have been recorded in the provinces of Udine (Machino, Y., 1996); (De Luise, G., 2006). For this reasons FVG regional authorities and Fisheries protection agency (ETP) have been long studying and monitoring native crayfish species. During the 2005 monitoring carried out on a large scale a significant decline of regional populations was detected; in 2007 the first documented reports revealed the presence of the Louisiana red swamp crayfish Procambarus clarkii, considered one of the most dangerous invasive allochthonous species for the conservation of biodiversity.

To achieve the goal of preserving the native crayfish the LIFE-RARITY project (LIFE 10 NAT /
IT / 000239) was undertaken with the main objective to preserve and enhance stocks of the native crayfish populations of *Austropotamobius pallipes* in the whole area of the Friuli Venezia Giulia (FVG) region. The project settles two specific and integrated objectives: 1) to contrast the diffusion of the Invasive Alien Species (IAS) *Procambarus clarkii*, representing a great threat to the regional population of *A. pallipes*; 2) to enhance indigenous crayfish populations through restocking and reintroduction programs. Given the complex genetic structure of *A. pallipes* in Italy, recovery operations must be preceded by verification of the extant genetic lineages in the area where we want to intervene.

The RARITY project ([www.life-rarity.eu](http://www.life-rarity.eu)) has therefore envisaged a set of genetic analysis, which are the focus of this PhD thesis, in order to provide useful information for the constitution of a native crayfish broodstock to be used for new offspring production in captivity and subsequent release into the wild. It is important, indeed, to create a broodstock containing in the genome most of the genetic diversity found in natural populations, whilst minimizing the risk of genetic pollution in the releasing sites through the introduction of genetically incompatible individuals. Indeed the introduction of individuals belonging to a different genetic lineage can lead to the long-term loss of the genetic identity of the native population, and in the short-term, to the failure of intervention and the waste of the allocated economic resources (Morpurgo et al., 2010)

### 1.2. The target species

#### 1.2.1. Biology

The White-clawed Crayfish *Austropotamobius pallipes* (Lereboullet, 1858) is one of four crayfish species indigenous to Europe and the most widespread Indigenous crayfish species (ICS) in Italy.

The body is smooth, generally brown to olive in colour with pale-coloured undersides to the claws (whence the specific epithet *pallipes*, meaning "pale-footed"). White-clawed Crayfish are rather slow growing compared to other astacids; they reach 9 cm total length and 40 g in 5 or more years, and an ultimate length of perhaps 12 cm (Holdich et al., 2002)

Crayfish grow by moulting their shell and increasing by about 10% in length before the new one hardens. Immature individuals may moult several times each year, but mature males usually moult twice (early and late summer) and reproductive females only once, in late summer. Adult males have larger claws than females and are more territorial, particularly in the mating season. Females develop a broader abdomen, which accommodates the brood. Males can also be distinguished from females by the specialised first two pairs of appendages on the undersides of
the abdomen. The appendages function like a plunger to introduce a white spermatophore mass onto the underside of the female during mating (Holdich, 2003).

Crayfish can live for more than 10 years, and usually reach sexual maturity after three to four years. Breeding takes place in autumn and early winter (September to November) when the water temperature drops below 10°C for an extended period. This time may vary with latitude and altitude, although changes in day-length may also be involved in triggering the response. Females overwinter with a clutch of eggs attached beneath the abdominal appendices (pleopods). The number of eggs carried may range from 20 to 160, but is usually less than 100 (Frankel, 1970; Holdich, 2003). Eggs hatch after an incubation period that lasts from 4 to 7 months, depending on thermal conditions of the water course, and juveniles remain attached to the mother for about two weeks before becoming independent at the beginning of summer; in their first year of life they are extremely prone to predation (Holdich, 2003).

1.2.2. Distribution, Habitat and Ecology

*Artemia pallipes* has a wide distribution range across Western Europe, covering the Iberian Peninsula, France, Great Britain, Ireland, Switzerland, Austria, Italy and the Adriatic coast of Slovenia and Croatia (Laurent, 1988).

The white-clawed crayfish inhabits streams, rivers and water courses with clean, cool and running waters, with a good oxygen content and relatively hard, mineral-rich waters on calcareous and rapidly weathering rocks. Waters containing this species tend to be in the pH range 7-9, with calcium levels ranging from 200 to 350 ppm (Holdich, 2003). Vertical banks and overhanging vegetation have been highlighted as important features in determining crayfish
abundance, (Naura and Robinson, 1998): riparian vegetation in fact plays an important role as a source of debris, provides shade, thus maintaining water temperature at an optimal value for crayfish, and offers protection from predators (Benvenuto et al., 2008). This freshwater species typically occupies cryptic habitats under rocks and submerged logs, among tree roots, algae and macrophytes. The white-clawed crayfish is largely nocturnal emerging to feed on a broad diet of detritus, animals and plants. This species is intolerant to pollution and hydrological change.

![Distribution range and conservation status of A. pallipes complex in Europe.](http://www.iucn.org)

**1.2.3. Major threats for conservation**

A list of major threats to crayfish would include: pollution (industrial, domestic, agricultural), habitat modification (dams, draining, dredging), illegal fishing and competition from non indigenous crayfish species (NICS), directly, indirectly or as vectors of diseases, climate change (droughts and floods) (Holdich, D.M. et al., 2009)

- **Pollution**: this species is highly susceptible to chemical alterations, to the extent that it is typically considered to be a good indicator of water quality (Brusconi et al., 2008; Scalici and Gibertini, 2005). It is particularly sensitive to heavy metals and pollutants in the runoff containing herbicides, pesticides and synthetic fertilizers used in agriculture. The
organic pollution from human settlements and animal breeding activities not only weakens the crayfish, making it more vulnerable to diseases, but also seriously damages its habitat by reducing the amount of oxygen in the water and altering the macroinvertebrate communities found in torrents (Holdich et al., 2002)

- **Habitat modification**: water abstraction for irrigation, domestic purposes, and watering livestock has had the effect of decreasing water availability seriously. This has led to the fragmentation of watercourses into small to medium sized pools characterized by very different micro-environments with respect to the original conditions, thus increasing the risk of chemical pollution especially when these pools are located near drains and farms. Finally, the fragmentation of watercourses has caused reproductive isolation into sub-populations with the consequent reduction in genetic diversity (Aquiloni et al., 2010). Local extinction can also be ascribed to physical alterations of the waterbodies, mostly caused by channelling the riverbed: partial or total concreting of the banks reduces or destroys the riparian vegetation, which represents a key factor for the survival of *A. pallipes* (Brusconi et al., 2008).

- **Overfishing and poaching**: fishing is one of the main causes of local extinctions of *A. pallipes* (Renai et al., 2006). Fishing leads to a drastic decrease in population size; if a population is already at low density because of other human induced threats (Scalici and Gibertini, 2005), its exploitation might reduce the genetic diversity (Bertocchi et al., 2008) thus increasing the vulnerability to both environmental stresses and random events. There are today regulations in Italy that restrict crayfish fishing, but in certain areas poaching continues as part of cultural traditions.

- **Introduction of Non Indigenous Crayfish Species (NICS)**: the most serious threat to our white-clawed crayfish comes from two introduced exotic species, Procambarus clarkii and Orconectes limosus, which are progressively replacing autochthonous populations and which pose a serious threat to their survival (Holdich, D.M. et al., 2009) These two American species act as a vector for the crayfish plague fungus *Aphanomyces astaci* Schikora, carrying it in its cuticle as a benign infection, which is lethal for native European crayfish species and can lead to massive mortality in the affected population in short periods of time (Kozubíková et al., 2008) They are also much more competitive than *Austropotamobius pallipes*, and have far more effective Behavioural and reproductive strategies.

- **Climate change**: climate changes results in an increase of extreme meteorological events such as droughts and floods (IPCC, 2012). The small, unpolluted hillside water courses
which provide the ideal habitat for these crayfish are often particularly vulnerable to drying up, especially in summer, because of their limited capacity. Reduction or drying up of tracts of the watercourse reduces the habitat available and exerts stress on crayfish populations. Stress derived from droughts and poor water quality can weaken crayfish and make them more vulnerable to epidemics (Richman et al., 2015).

1.2.4. Taxonomic status and genetic studies

Due to the high morphological heterogeneity, taxonomy of white clawed crayfish has gone through numerous revisions over the last five decades (Bott, R., 1950; Karaman, M.S., 1962; Starobogatov, Y.I., 1995) and is still under debate. In the last decades genetic studies were employed to investigate its taxonomic status using Mitochondrial DNA, which is a widely used molecular marker in phylogenetic and phylogeographic studies due to its maternal inheritance and its lack of recombination (Avise, J.C., 1994).

Fig.3. Map of geographic distribution of *A. pallipes* and *A. italicus* in Italy. Symbols on the map indicate: C= *A. i. carinthiacus*; It= *A. i. italicus*, M= *A. i. meridionalis*, Ca= *A. i. carsicus*, P= *A. pallipes*, pc= *A. pallipes* and *A. i. carinthiacus* mixed population; PV=Padan-Venetian ichthyogeographic district; T=Tuscan-Latium district; S=Southern Italy district. (modified from Fratini, 2005).
The first genetic studies on the taxonomic status of the white clawed crayfish used the mitochondrial marker 16S rDNA in combination with morphological characters and suggested that *A. pallipes* is a species complex and should be subdivided into two different species (Grandjean et al., 2002, 2000): *A. pallipes* mainly distributed across Western Europe (France, Germany, and the British Isles), and *A. italicus* (Faxon, 1914), although the name is still being discussed, associated to a southern Europe distribution (Italy, the Adriatic basins in the Balkans and the Iberian Peninsula. As shown later by genetic analysis, in the Italian peninsula the two species *A. pallipes* and *A. italicus* live in sympatry in the Apennine Ligure and in the Province of Alessandria (Zaccara et al., 2004). The 16SrDNA-based taxonomy proposed by Grandjean et al., (2000; 2002) envisaged three further subspecies within the *A. italicus* species, all found in the Italian peninsula: *A. i. italicus* in the North-Central Apennines, *A. i. carsicus* in North-Eastern Italy, *A. i. carinthiacus* in the Central and North Western regions. Fratini (2005) conducted an extensive sampling throughout the Italian peninsula and described a further subspecies: *A.i. meridionalis* in Southern Italy.

In the last decade this marker was applied to further investigate taxonomic status of *A. pallipes complex*. Iaconelli (Iaconelli, 2001) found that in *A. pallipes* s. lato mitochondrial cytochrome oxidase subunit one (COI) gene sequences are more variable than 16S rDNA sequences. Indeed the COI gene has proven to be a suitable marker to address finer-scale phylogenetic and phylogeographic studies in closely related taxa. (Hajibabaei et al., 2007; Verovnik et al., 2004; Hebert et al., 2003). Zaccara et al. (2005) investigated the diversity of *A. italicus* using the COI mitochondrial gene for the populations of Po River drainage basin, and in a larger study Trontelj et al. (2005), used the COI gene sequences to investigate phylogenetic and phylogeographic relationships for the entire European freshwater crayfish genus Austropotamobius. Although the results are not in complete agreement with the subdivision of *A. pallipes* s. lato into *A. pallipes* and *A. italicus*, different haplogroups were identify within the clade: Western Europe, corresponding to the taxon *A. pallipes* sensu Grandjean et al. (2000a); all other haplogroups of the *A. pallipes* s. lato clade supposedly conform *A. italicus* sensu Grandjean et al. (2000a) and are: North Western Italy, Istra 1, South Eastern Alps-W Balkans, Appenine and Istra 2. *complex* genetic diversity patterns were even more evident when the genetic analysis was extended to populations from different Italian regions particularly in the North Western and Central regions (Bertocchi et al., 2008; Cautadella et al., 2010; Chiesa et al., 2010; Stefani et al., 2011), revealing a strong genetic structure on fine geographical scale.

In FVG region several detailed studies exist on biology, ecology, behaviour and distribution of
the white-clawed crayfish (De Luise, 2006), but little is known about the phylogenetic status and genetic composition of local crayfish populations. Previous genetic studies, including some samples from FVG, gave a patchy and contrasting picture of this area. Fratini (2005) analyzed 2 individuals from the Rosandra stream (south eastern FVG) for the 16S rDNA gene and assigned them to the A.i. carsicus subspecies, in accordance with the described geographic range of such clade in the NE Italy, as it is found also in Veneto and Southern Trentino (NE Italy). Trontelj (2005) conducted a study with the mtDNA COI marker in the nearby areas of Western Balkans and Austria, including some sequences from FVG region available from a previous unpublished study by Iaconelli (2001). where FVG populations were assigned to the SEA-W Balkans haplogroup, also present in southern Austria and in the Eastern Adriatic coastal area, to the south. A first attempt to to link the COI genetic structure with the taxonomy of the white clawed crayfish based on the 16S rRNA was done by Cataudella et al. (2010) in the Marches region (Central Italy), where a few samples from FVG individuals were included. The comparison between 16S RNA and COI markers showed that: A. i. meridionalis status was shared by several COI clusters defined by Trontelj et al. (2005); in particular individuals belonging to both COI Southern Alps /Western Balkans (FVG) and Appenine lineages (central-southern Italy) share the same 16S RNA sequence and thus the same taxonomic status.

1.3. Conservation genetics

The relevance of genetic factors and evolutionary processes to conservation biology has been recognised for over 30 years (Frankel, 1970, 1974); (Frankel and Soulè, ME, 1981). Since this time, the field of conservation genetics has burgeoned and genetic information (particularly from the mitochondrial genome and nuclear microsatellite DNA) has been used to address problems of conservation significance across a broad range of evolutionary scales from systematics and taxonomy, to hybridisation, the delineation of conservation units, detection of fragmentation and metapopulation structure, kinship and individual identification. The major distinction between conservation genetics and other population genetics research is the explicit objective of contributing to the preservation and recovery of threatened populations and species (Frankham et al., 2002). Thus, the field of conservation genetics applies the study and theory of population genetics to generate recommendations for maintaining genetic diversity in threatened species (Frankham et al., 2004).

Genetic variation provides the basis for selection, adaptation and speciation (Amos and Harwood 1998). Therefore, as genetic variation decreases so does adaptive potential, or the capacity to
adapt to changing environmental conditions (Frankham, 2005). Conservation of genetic variability is important to the overall health of populations because decreased genetic variability leads to increased levels of inbreeding and decreased fitness.

1.3.1. Basics of population genetic theory

Populations evolve through the action of natural forces: selection, mutation, migration (gene flow) and chance (genetic drift) (Hartl et al., 1997) The role of this factors can be summarized as follows:

- mutation is the source of all genetic diversity, but is a weak evolutionary force over the short term, as mutation rates are generally very low.
- migration (gene flow) reduces differences among populations generated by mutation, selection and chance.
- Selection is the only force causing evolutionary changes that better adapt populations to their environment.
- When inherited characteristics determine who will survive and who will not, the process is called natural selection. When random factors determine who will survive, the process is called genetic drift.; gene frequencies in real populations change randomly with time through genetic drift.

The unifying concept of population genetics theory is the Hardy-Weinberg law that predicts how gene frequencies will be transmitted from generation to generation given a specific set of assumptions (Templeton, 2006). In large, random mating populations, allele and genotype frequencies at an autosomal locus reach equilibrium after one generation (Hardy-Weinberg equilibrium, HWE) and will remain the same from generation to generation, when there are no perturbing forces (no mutation, migration or selection). Deviations from HWE genotype frequencies are highly informative, allowing us to detect the factors underlying the departure from equilibrium: inbreeding, population fragmentation, migration genetic drift and selection.

1.3.2. Applications of Conservation genetics

1.3.2.1. Management of small populations

Species and populations of conservation concern have small or declining population sizes. Surveillance of small populations is critical, because they are particularly sensitive to change. Random or unpredictable events such as natural catastrophes, environmental changes, or genetic
mutations can cause a sudden decrease in population size. (Frankham et al., 2002) When the population of a species is small to begin with, further reduction of their remaining numbers can sharply reduce genetic diversity. Small, isolated populations suffer accelerated inbreeding and loss of genetic diversity leading to reduced reproductive fitness (inbreeding depression) and reduced ability to evolve in response to environmental change. Small populations are also more sensitive to genetic drift, as well as the problems that come with geographic isolation and establishing a new population from only a few individuals (founder effect). (Frankham, 2005). Each of these factors affects which individuals will give rise to the next generation, and therefore which alleles will be passed on.

• **Genetic drift**

Genetic drift is the change in the frequency of a gene variant (allele) in a population due to random sampling. The alleles in the offspring are a sample of those in the parents, and chance has a role in determining whether a given individual survives and reproduces (Hartl et al., 1997). Real populations rarely exist under the rigid conditions of the Hardy-Weinberg Equilibrium. Instead, gene frequencies in real populations change randomly with time through genetic drift. Evolution due to genetic drift is not caused by environmental or other kinds of stresses on individuals, and the resulting random changes can be detrimental, neutral, or beneficial to the reproductive success of the following generation (Frankham et al., 2002). Chance effects normally have minor impacts on the genetic composition of large populations compared to the entire gene pool and are often overshadowed by natural selection. However genetic drift within small populations has consequences of major significance in evolution and conservation, such as the loss of genetic diversity and fixation of alleles within populations, with consequent reduction in evolutionary potential. Population bottlenecks occur when a population’s size is reduced for at least one generation. Because genetic drift acts more quickly to reduce genetic variation in small populations, undergoing a bottleneck can dramatically reduce a population’s genetic variation, even if the bottleneck doesn’t last for very many generations (Nei et al., 1975). The same effect is observed in the founder effect, when a few individuals in a population colonize a new location that is separate from the original population undergoing a genetic diversity loss due to random sampling of the original population. The founder effect is an extreme example of genetic drift. Genes occurring at a certain frequency in the larger population will occur at a different frequency in a smaller subset of that population just by chance.

• **Inbreeding**

In small closed populations, mating among individuals (Inbreeding) is inevitable. Inbreeding results in reduction of heterozigosity, which can increase the chances of offspring being affected
by recessive or deleterious traits (Hedrick and Kalinowski, 2000). This generally leads to a decreased biological fitness of a population (called inbreeding depression), which reduces its ability to survive and reproduce (Charlesworth, 1987).

• **Population structure and fragmentation**

  Genetic variation within a species is often spatially structured if the available habitat is not continuous or varies across the distributional range. (Hanski, 1998) Habitat fragmentation is one of the central concerns of conservation genetics as it reduces the total area of suitable habitat available and restricts gene flow between patches, inevitably leading to reductions in population size and increased differentiation through genetic drift. Fragmentation increases the risk of population extinction through genetic, demographic and environmental factors (Frankham, 2005). Human-induced habitat fragmentation through activities such as land-clearing, urbanisation, forestry, agriculture, mining and dam construction is widely acknowledged as a key threatening process to the long-term persistence of threatened populations, species and ecosystems (Vié et al., 2009). An understanding of the population structure of threatened species is critical to their effective management and conservation. Translocations and breeding programs aimed to supplement diminished wild populations may be detrimental if the source population is genetically distinct from the recipient population (Edmands and Timmerman, 2003); (Eldridge and Naish, 2007).

• **Selection**

  Selection is less effective in small than large populations. In large populations selection typically dominates allele frequencies changes for alleles subject to natural selection. Conversely in small populations genetic drift is usually the dominant force causing allele frequencies change, even for alleles subject to natural selection. An important implication of this is that deleterious alleles are less likely to be removed by natural selection and may even become fixed (Frankham et al., 2002).

1.3.2.2. **Solve taxonomic uncertainties**

To properly appreciate and understand biodiversity, we must have a sense of phylogenetic structure of the taxa involved. This applies to broad levels of organization (soil bacteria, plants, animals) as well as to smaller taxonomic units (populations within species). Traditionally morphology was used for taxonomic classification, however, some species are morphologically indistinct due to recent separation or limited morphological divergence despite speciation and molecular studies have uncovered many cases of cryptic speciation ((Musyl and Keenan, 1996); (Burbidge et al., 2003); (Cook et al., 2008)). Molecular systematic approaches have been of great
use since many new techniques can be applied without harming wild individuals, and can even be applied to museum skins for historical comparisons (Irestedt et al., 2006). Taxonomic status must be accurately established so that endangered species are not denied protection or on the contrary resources wasted on abundant species.

1.3.2.3. Definition of Management units and ESUs
Evolutionary lineages within species may require separate conservation management where they are historically isolated or adaptively distinct from other populations of the same species (Crandall et al., 2000). Moritz ((Moritz, 1994) suggested that molecular markers could be applied to define intraspecific conservation units. Evolutionarily significant units (ESUs) were defined as those demonstrating reciprocal monophyly for mitochondrial DNA (mtDNA) and significant allele frequency differences at nuclear loci. Despite various definitions, a uniform agreement might be that an ESU is a lineage demonstrating highly restricted gene flow from other such lineages within the higher organizational level (lineage) of the species (Fraser and Bernatchez, 2001). Another relevant level of concern is a Management Unit (MU), defined as those populations showing genetic divergence at mtDNA and/or nuclear DNA, but do not necessarily show fixed differences between populations (Palsbøll et al., 2007) Hence several MUs may exist within an ESU. Management units are demographically independent populations that require management but need not necessarily be preserved as separate entities. ESUs represent evolutionary lineages requiring separate management, therefore translocations of individuals among ESUs should be avoided (Moritz, 1999).

1.3.3. The tools of conservation genetics: molecular markers
Molecular markers represent the tools that conservation scientists routinely use to assay genetic variation in threatened species. Neutral markers, such as non-coding mitochondrial regions and nuclear microsatellite DNA, are most commonly applied to measure genetic variation by comparative levels of polymorphism, heterozygosity and allelic diversity (Beebee and Rowe, 2004)

The choice of the most appropriate molecular marker in conservation genetics is driven by the definition of clear and focused questions to be answered. The main addressed questions of this study are:

- define the taxonomic placement of regional A. pallipes-complex populations in FVG;
- the evaluation of genetic diversity as a means of estimating populations' health status;
- the identification and definition of Evolutionary significant units and Management Units.
1.3.3.1. Mitochondrial DNA (mtDNA)

The mitochondrial genome comprises a circular ‘chromosome’ of DNA. Animal mtDNA ordinarily contain 36 or 37 genes; two for ribosomal RNAs, 22 for tRNAs and 12 or 13 for subunits of multimeric proteins of the inner mitochondrial membrane (Boore, 1999). In addition, there is a non-coding sequence termed the control region (CR) due to its role in replication and transcription of mtDNA molecules. A typical characteristic of mtDNA is maternal inheritance that makes it suitable for reconstruction of phylogenetic relationships, because the smaller effective population size gives consequently a shorter expected time to reciprocal monophyly between taxa. (Moritz, 1994) Since mtDNA lacks of recombination mutations are the only cause of its variability and mutations accumulated since female ancestors were last shared, originate different mtDNA genotypes, called haplotypes (Avise, 2009). Mitochondrial DNA is histone-free, has limited repair ability, and therefore has a relatively high mutation fixation rate (5–10 times that of scnDNA) (Jansen, 2000). Although mtDNA has evolved faster than the nuclear genome, the rate of evolution is different for different regions of mtDNA and has been used to examine various phylogenetic relationships: 12s rDNA is highly conserved and has been employed to illustrate phylogeny of higher categorical levels such as in phyla or subphyla. 16S rDNA is usually used for phylogenetic studies at mid-categorical levels such as in families or genera (Wan et al., 2004). Compared to 12s and 16S rDNAs, the mitochondrial protein-coding genes, such as the Cytochrome oxidase (CO), evolve much faster and are powerful markers for inferring evolution history in lower categorical levels such as species and subspecies. These features of mtDNA in phylogeny makes it suitable for resolving taxonomic uncertainties in conservation genetics (Hebert et al., 2003). Therefore mtDNA was chosen to define the taxonomic status of regional crayfish populations and because, when utilizing mtDNA to define species priority, one should use multiple loci rather than a single gene in order to infer correct species identity, we selected two different mtDNA markers: 16S rDNA and Cytochrome oxidase subunit I (COI). Moreover the choice of these two markers allows us to make the widest comparison of our data with previous studies on the *A. pallipes* complex, discussed in par. 1.2.4.

- **16S rDNA**

The gene coding for the 16S rna of the large ribosomal subunit are referred to as 16S rDNA and are used in reconstructing phylogenies, due to the slow rates of evolution of this region of the gene (Weisburg et al., 1991). In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature...
sequences useful for bacterial identification (FOX et al., 1977) MtDNA 16S has been widely used for establishing the evolutionary relationships of genera and species in different animal taxa (Dong, 2014; Hajibabaei et al., 2007)

- **Cytochrome c oxidase subunit 1 (COI)**

The taxonomy and systematics of freshwater crayfish is still in the process of change and development, due to the enormous morphological plasticity displayed by decapod crustaceans (Burnham and Dawkins, 2013). Recently a big effort has been made to compile regional databases that identify and analyse the extent and patterns of decapod diversity throughout the world through cytochrome c oxidase subunit 1 (COI) barcoding analysis (da Silva et al., 2011). This region of the mitochondrial DNA (mtDNA) has been proposed as a barcoding tool, or at least to confirm species delimitation for taxonomic, ecological and evolutionary studies (Hebert et al., 2003) Species identification relies on the observation that barcode sequence divergence within species is typically much lower than the divergence exhibited between species, defined “barcoding gap”.

1.3.2.2. Microsatellites

A major focus of conservation genetics is on contemporary genetic structuring in small populations, and as such requires DNA markers that have a high evolutionary rate. Microsatellites are tandemly repeated motifs of 1–6 nucleotides dispersed throughout the eukaryotic nuclear genome and their polymorphisms are the result of variations in the number of tandem repeats of the core sequence.

Currently, microsatellites are the most popular markers in livestock genetic characterization studies (Selkoe and Toonen, 2006). Declines in genetic diversity can be detected by sensitive genetic markers, such as microsatellites, that can reveal reductions in heterozygosity and allelic diversity in small and fragmented populations. Indeed their high mutation rate and codominant nature permit the estimation of within and between population genetic diversity, and genetic hybridization/admixture among taxa even if they are closely related. Furthermore it is possible to detect recent demographic events (bottlenecks, drift) and the degree of habitat fragmentation (through estimates of gene flow and differentiation), shaping the extant genetic structure of populations (Bruford et al., 1996). This kind of genetic data can be particularly useful in conservation genetics studies aiming at identifying priority populations for conservation.

The first studies employing microsatellite loci to investigate genetic diversity in the white clawed crayfish were carried out in France by Gouin and colleagues (Gouin et al., 2001, 2000) revealing
no genetic differentiation between different locations along a brook suggesting that the species had higher dispersal ability than previously thought. Opposite results were obtained by the analysis of five microsatellite loci in an extensive study of French populations (Gouin, 2006), detecting significant differentiation among river drainages as well as within rivers. Two strongly differentiated groups, corresponding to northern and southern French populations, were proposed as two evolutionarily significant units (ESU) for *A. pallipes* in France, to be managed separately. Recently Gouin (Gouin et al., 2011) reported a strong positive correlation between population density estimates of mature individuals and levels of genetic variability in 10 populations from western France, highlighting the importance of demographic factors, such as bottlenecks and genetic drift on *A. pallipes* populations. Up to date in Italy only few studies applied microsatellite markers to investigate genetic diversity of *A. pallipes* at the local scale. Baric (Baric et al., 2005), by analysing four microsatellite DNA loci in South Tyrol populations, found not only substantial genetic structure among populations, but also moderate genetic variability within populations, in contrast with extremely low diversity displayed by previous mtDNA analysis. Bertocchi (Bertocchi et al., 2008) applied an integrated approach using the mitochondrial 16S rDNA gene and nuclear microsatellite loci in five river basins in Tuscany, detecting low intra-population genetic variability with high levels of inbreeding and high inter-population genetic divergence attributed to strong habitat fragmentation. Opposite results were obtained by Chiesa et al. (Chiesa et al., 2011), that combined mtDNA COI sequences with AFLP technique (nuclear marker) to assess genetic diversity within and among Austropotamobius populations throughout Italian peninsula, finding quite contrasting evidence: the COI marker revealed a strong geographic structure while nuclear markers showed unexpected low levels of genetic differentiation and the absence of any geographic structure. These studies suggest that there may be large discrepancies between results depending upon whether information is obtained by mtDNA or nuclear markers. This discordance stresses the importance of collecting data from both types of genetic markers before interpreting data and making recommendations for the conservation and management of natural populations.

A main objective of the present study was to identify potential ESU for management purposes; ESUs have been distinguished from other populations in some species based on mtDNA (Moritz, 1994) and/or nuclear DNA. Because unrelated phenomena are likely to leave their footprints on genetic structure, clearly there is a need to combine different kinds of markers to identify a true ESU. Therefore to evaluate genetic diversity within and among populations and identify potential ESUs, I chose to combine the use of two mtDNA markers (COI and 16S) with six polymorphic microsatellite loci.
2. Aim of the work

The main objective of this study was to provide new knowledge on white-clawed crayfish populations in FVG to create “genetic maps” that can be used as valuable data for restocking practices and conservation programs in FVG.

A combined approach of mitochondrial and nuclear markers (microsatellites) was employed in a regional-scale genetic monitoring of populations in order to:

- define the taxonomical status of wild populations through phylogenetic analysis of the two mitochondrial loci (16S and COI)
- assess the overall “health” status of populations through the estimation of genetic diversity levels within and between populations, and attempt to understand major causes of current genetic structure (mainly through microsatellites).
- Identify strongly differentiated population groups representing Evolutionarily Significant Units (ESU) to be treated as separate management units (mtDNA and microsatellites).
3. Materials and methods

3.1. Sampling
For the present study 506 white-clawed crayfish specimen from 58 localities in the Friuli Venezia Giulia (FVG) region were sampled during the spring-summer period between 2012-2013, from the 6 main hydrographic drainages: Tagliamento, Livenza, Isonzo, Cormor, Levantine basin and Stella (tab.1; fig.4). One pereopod per individual was removed and preserved in absolute ethanol for further analysis while all individuals were returned to the river after sampling. only male individuals were sampled in order to avoid creating excessive stress in the females during the spawning season.

Sampling for genetic analysis was carried out on the last day of a week-long biological monitoring, so as not to alter the behaviour of crayfish and their potential catching with fish traps, which would have jeopardized a proper estimate of the population size with the marking/recapture technique. In some cases a significant decrease in crayfish captures was observed during the week, affecting sample size available for genetic analysis. Fig.4. Map of Sampling sites of white-clawed crayfish populations in FVG and Lombardia-Trentino.

In the sites where literature indicated the possible joint presence of *A. pallipes* and *A. torrentium* (sites 52, 54 and 58 in north eastern Tagliamneto) the monitoring protocol was adjusted and all individuals captured were first sampled for genetic analysis, to assess the presence of both species with molecular tools. 59 specimen were sampled from these stations but neither genetic analysis nor taxonomic recognition revealed the presence of *A. torrentium*. 
Fig. 4. Map of Friuli Venezia Giulia and Trentino/Lumbardy sampling locations.
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<th>Station</th>
<th>Drainage Basin</th>
<th>N</th>
<th>Code</th>
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Tab.1. Sampling locations and number of individuals (N) included in present study. For each location major drainage basins are indicated.

3.2. DNA isolation, amplification and capillary electrophoresis

Genomic DNA was isolated using the EZNA® Tissue DNA Kit (Omega Bio-tek, Norcross, GA)
Pcr amplifications for the two mitochondrial markers (16S and COI) were carried out in 25 μL reactions containing: 1X PCR buffer, 2 mM MgCl2, 0.5 μM of each primer, 0.2 of each dNTP, 1 U Taq polymerase (Promega, Fitchburg, WI) and about 20 ng of template DNA. The 5’ region of COI was amplified using primers ORCO1F (5’- AACGCAACGATGATTTTTTCTAC-3’; (Taylor and Hardman, 2002)) and APACOI1H (5’- ATAGCGACTATAGCATAAAATTATC-3’; (Pedraza-Lara et al., 2010)) by applying 35 cycles of 30 s at 94ºC, 30 s at 48 ºC and 60 s at 72 ºC, after an initial 3 min denaturation at 94 ºC. Amplification of a 500 bp long fragment of rDNA16S was carried out using primers 16Sbr 5’-CCGGTCTGAACCTYCTACGT-3’, (Palumbi et al., 1991) and AP_16SWf 5’-TTTCTGCCTGTTTAACAAAAACAT-3’, (Pedraza-Lara et al., 2011) present study) with the following pcr conditions: initial 3 min denaturation at 94 ºC, 30 s at 94ºC, 30 s at 52ºC and 60 s at 72 ºC (30 cycles), followed by 5 min final extension at 72 ºC. PCR products were visualized in 1.5 % agarose gel stained with Green Gel Safe (Fisher Mol. Biol.). Sanger sequencing was performed in the forward direction by the Applied Genomic Institute (IGA) of Udine.

The microsatellite (SSR) study included six loci (tab.2): two developed for A. pallipes and four recently developed by Pedraza et al (2011), specifically for A. italicus. SSR loci were amplified in two multiplex reactions: multiplex 1 included loci Ait 1, ait 5, Ait 9, Ait 11 and multiplex 2 included loci AP2 and AP3. KAPA 2G Fast Multiplex PCR Kit was used to amplify all loci, in a final reaction volume of 10 μL, containing 5 ng of total DNA 1X pcr master mix, 0.1 μM of Ait11 and Ap3 primers, and 0.2 μM of all other primers. Amplification conditions were 95 for 3 minutes, followed by 10 cycles of 95°C for 30 s, 65°C for 30 s with a touchdown temperature gradient of -0.5°C by cycle, then 30 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 30 s; and 1 cycle at 72°C for 10 minutes.

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<th>Reverse Primer (5’–3’)</th>
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<th>M</th>
<th>N alleles</th>
<th>Size range (bp)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ap2</td>
<td>(CA)$_{16}$</td>
<td>TTCGATAAACGGTGCCTACGT</td>
<td>TCAGACTTGGCCATTGAAG</td>
<td>FAM</td>
<td>2</td>
<td>19</td>
<td>124-252</td>
<td>Gouin, 2000</td>
</tr>
<tr>
<td>Ap3</td>
<td>(CA)$_{14}$</td>
<td>CGCCTATCTACCTTTCTTCTC</td>
<td>GGACTTGGGAGCGCCTGTC</td>
<td>HEX</td>
<td>2</td>
<td>19</td>
<td>126-238</td>
<td>Gouin, 2000</td>
</tr>
<tr>
<td>Aital1</td>
<td>(CA)$_{12}$</td>
<td>ACAGATCTCTGCTGCTGCTGT</td>
<td>GCCAAAGCACAACCTAGTGA</td>
<td>JOE</td>
<td>1</td>
<td>17</td>
<td>177-209</td>
<td>Pedraza-Lara, 2011</td>
</tr>
<tr>
<td>Aital5</td>
<td>(ACAS)$_{15}$</td>
<td>CACTACCCACACGGAGATGTG</td>
<td>TGGCGATATGATGCTACTG</td>
<td>TAMRA</td>
<td>1</td>
<td>18</td>
<td>231-283</td>
<td>Pedraza-Lara, 2011</td>
</tr>
<tr>
<td>Aital9</td>
<td>(CASA)$_{6}$</td>
<td>TTCACATATGCTGATAGCTCC</td>
<td>TCTGGCAGACCTGCTACTCC</td>
<td>JOE</td>
<td>1</td>
<td>17</td>
<td>329-379</td>
<td>Pedraza-Lara, 2011</td>
</tr>
<tr>
<td>Aital11</td>
<td>(GACA)$_{11}$</td>
<td>GACAAAATGCGCGCCCTAAC</td>
<td>ATGTGTGCTGTTTAAAGCCTCG</td>
<td>FAM</td>
<td>1</td>
<td>9</td>
<td>193-233</td>
<td>Pedraza-Lara, 2011</td>
</tr>
</tbody>
</table>
Tab.2. Detailed description of microsatellite loci used in present study; locus name, repeat motifs, forward and reverse primer sequences, fluorescent dye used, multiplex reaction (M), number of alleles and references are reported for each locus.

SSR loci were amplified using a reverse fluorescent labelled primer and pcr products were run with the internal size standard GeneScan 500 ROX (Applied Biosystemms, USA) on a 3130 DNA Analyser (Applied Biosystems, USA). Allele size was determined through Gene Mapper v4.0 (Applied Biosystems, USA) and visually checked to assess the correctness of scoring.

3.3. Data analysis

3.3.1. MtDNA

3.3.1.1. Dataset organization and sequence analysis

Since previous works have not always analysed the same mitochondrial markers, for the phylogenetic analysis I decided to use both mitochondrial markers separately, to infer the most complete phylogenetic framework as possible, and to be able to make the widest comparison with literature. We included in this analysis 29 samples from North Eastern Italy kindly provided by the Istituto zooprofilattico delle Venezie: 12 samples from Trentino and 17 from Lombardy. Genetic diversity and phylogeographical analysis were conducted only on COI dataset as it provided higher variation to study population diversity and allow comparison with more recent studies. Finally to detect population genetic structure including simultaneous analysis from geographical data, we merged the two datasets (COI and 16S rDna) in a Bayesian clustering analysis with the software GENELAND.

3.3.1.2. Phylogenetic analysis

The phylogenetic analysis was conducted to assign the crayfish populations of FVG region to the different subspecies of the Austropotamobius pallipes species complex identified in previous studies (Fratini et al., 2005; Grandjean et al., 2000). 33 additional sequences (16S) and 38 (COI) were added from genbank to the alignments, representing haplotypes of the major lineages of the A. pallipes complex; a sequence from A. torrentium was used as the outgroup. At first analysis were conducted on all obtained sequences; thereafter, representatives of the different haplotypes
were identified using the program Arlequin 3.5 (Excoffier and Lischer, 2010), and subsequent analyzes were conducted on haplotypes.

The best fitting substitution models were chosen with jModeltest 0.0.1 (Posada, 2008) using the Akaike information criterion corrected for small sample size (AICc) and the Bayesian information criterion (BIC). The chosen model for 16S was the T92+G model with a gamma distribution shape parameter 0.54 of and the HKY+G with a gamma distribution shape parameter of 0.21 for COI. Phylogenetic trees of the haplotypes were constructed using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) from two independent runs with four chains over 15 000 000 generations, sampled every 1500th generation and excluding the first 2000 trees as burn-in. Since the T92+G model is not implemented in MrBayes, the program was run with the closest over-parameterized model, the HKY+G model, for both markers. The burn-in period was determined as the set of trees saved prior to log likelihood stabilization and convergence as estimated using Tracer v1.5 (Rambaut and Drummond, 2007).

3.3.1.3. Genetic diversity

The genetic diversity statistics were conducted using the COI dataset only for the FVG samples (n= 349) samples. Geographical regions were determined according to the river basins sampled. Standard estimates of genetic diversity and sequence polymorphism were examined using the program DnaSP v5.10.1 (Librado and Rozas, 2009) according to the proposed regionalisation. We generated an haplotype distribution and calculated, for each region, the number of polymorphic segregating sites (S), the number of haplotypes (H) and private haplotypes (Hp), haplotype diversity (Hd) and the average number of nucleotide differences (k). To account for differences in sample size within each subset, we estimated haplotype richness (Hr) by rarefaction with Hurlbert method (1971) implemented in the program HP-rare v. 1.0 (Kalinowski, 2005)

3.3.1.4. Phylogeographic analysis

Use of networks for shallow divergence topologies are better suited for the analysis of intraspecific phylogeographic relationships because closely related haplotypes are best represented in a multifurcating network rather than requiring occupation of tip positions in a phylogenetic tree (Morrison, 2005; Posada and Crandall, 2001).

Two separate Haplotype network analysis were conducted on COI dataset: one of the whole
species complex, including sequences from genebank and the Trentino-Lombardia samples, and a second only of the FVG samples to better visualize haplotype relatedness and geographical haplotype distribution of the local crayfish populations. We used median-joining algorithms (Bandelt et al., 1999) implemented in NETWORK v4.6.11 (Fluxus-Engineering) using epsilon=0 and unrooted settings.

3.3.1.5. Genetic structure and differentiation

Natural populations of living organisms often have complex histories consisting of phases of expansion and decline, and the migratory patterns within them may fluctuate over space and time. When parts of a population become relatively isolated, e.g., due to geographical barriers, stochastic forces reshape certain DNA characteristics of the individuals over generations such that they reflect the restricted migration and mating/reproduction patterns (Corander et al., 2008). Such populations are typically termed as genetically structured and they may be statistically represented in terms of several clusters between which DNA variations differ clearly from each other. When detailed knowledge of the ancestry of a natural population is lacking, the DNA characteristics of a sample of current generation individuals often provide a wealth of information in this respect. Several statistical approaches exist to detect patterns of population structure, such as F-statistics and analysis of molecular variance (AMOVA), model-based approaches (bayesian clustering algorithms) and exploratory approaches (PCA, PCoA, MDS and others).

- **Model-based approaches**

We used first a model-based approach through two Bayesian clustering methods implemented in the softwares STRUCTURE (Pritchard et al., 2000) and GENELAND (Guillot et al., 2005) to identify population groups and eventually detect ESUs and Management units for future conservation strategies. These methods differs from other statistical procedures for estimating genetic subdivision, such as F-statistics or the analysis of molecular variance that quantify the divergence among predefined subpopulations (Wright 1951; Excoffier et al. 1992). Instead these softwares assumes that there are \( K \) clusters (\( K \) is unknown), characterized by a set of allele frequencies at each locus, that can be interpreted as different gene pools from which populations originated. The Bayesian model implemented evaluates the presence of Hardy Weinberg and linkage disequilibrium in the sample and try to group populations in order to minimize the differentiation within each group, whilst maximizing differences between the groups. Among the Bayesian clustering methods, structure is the most widely used, but does not handle mtDNA
haploid data, so it could only be used for SSR analysis.  
We examined genetic structure among the COI and 16S merged datasets using clustering analyses implemented in GENELAND v. 3.1.4 (Guillot et al., 2005). This software implements an algorithm attempting to cluster samples on the basis of both genetic and geographic information. Bayesian statistics and spatial data for each individual in the form of x and y coordinates to infer genetic clusters, assuming that the overall sample consists of K clusters that are in Hardy-Weinberg and linkage equilibrium, and then assign each individual to these genetic clusters. The geographic information is accounted for at the Bayesian prior level in such a way that clusters corresponding to spatially organized groups are considered more likely than those corresponding to completely random spatial patterns. The benefit of using a spatial prior (presumably more informative than a nonspatial prior) is to get more accurate inferences and to explicitly infer the spatial borders between inferred clusters. We ran the MCMC five times (to verify the consistency of the results), allowing K to vary, with the following parameters: 200 000 MCMC iterations, maximum rate of Poisson process fixed to 500, minimum K fixed to 1, maximum K fixed to 30; the first 40,000 iterations were discarded as burn-in. Once k was determined, the model with the fixed number of populations K was run five times, each for 200,000 MCMC iterations. In post-processing of the chains, the first 40,000 iterations were discarded and maps of posterior probabilities of population membership were obtained and compared among the independent MCMC runs.

- **F statistics and Analysis of Molecular Variance (AMOVA)**

The genetic differentiation of populations is a key parameter in population genetic investigations. The most widely used metric of genetic differentiation has been Wright’s (Wright, 1965, 1943) fixation index, $F_{st}$, which was developed as part of a set of hierarchical parameters ($F_{st}$, FIS, and FIT) to assess the way in which genetic variation is hierarchically partitioned in natural populations. Modern analogues to $F_{st}$ include GST, derived explicitly to deal with multiple alleles at a locus (Nei, 1987, 1973) $\Theta$ (Cockerham, 1973, 1969); (Weir and Cockerham, 1984), and $\Phi_{ST}$ (Excoffier et al., 1992).

The most widely used index is still $F_{st}$ (Weir and Basten, 1990), which indirectly measure the degree of genetic differentiation of subpopulations through calculation of the standardized variances in allele frequencies among populations. Statistical significance can be calculated for the $F_{st}$ values between pairs of populations (Weir and Cockerham, 1984) to test the null hypothesis of a lack of genetic differentiation between populations and, therefore, the partitioning of genetic diversity. Arlequin v. 3.5 software (Excoffier and Lischer, 2010) was used to calculate the overall and pairwise genetic differentiation through the calculation of fixation
indeces. I used ΦST, which is an analogue of F estimates of genetic distance, accounting for the inter-haplotype sequence divergence. The significance of fixation indexes was tested by comparing the observed distribution with a null distribution generated by 1000 permutations, in which individuals were redistributed randomly into samples, as described in Excoffier et al. (1992). A hierarchical analysis of molecular variance (AMOVA) was performed with ARLEQUIN v.3.5 (Excoffier and Lischer, 2010) to assess the distribution of diversity and to compare the values for $F_{ct}$ (difference among groups), $F_{sc}$ (difference among populations within groups), and $F_{st}$ (differences among all populations). In this analysis, ‘groups’ refers to data grouped according to river basins (AMOVA 2) and to GENELAND output clusters (AMOVA 3). Computations were performed with 10,000 permutations to test for significance, within and among groups.

3.3.2. Microsatellites

3.3.2.1. Detection of allele scoring errors
Genotyping errors may arise when primer-site mutations result in non-amplified alleles (null alleles), resulting in an apparent excess of homozygotes, since heterozygotes for a null allele and another allele appear to be homozygotes; PCR artefacts can cause large allele drop-out (i.e. short allele dominance) and stuttering (observed as multiple artefact peaks preceding the true allele peak. Such errors can cause deviations from Hardy-Weinberg equilibrium, and may bias both spatial and temporal population genetic analyses. Null alleles, stuttering, and allelic drop-out were tested with Micro-Checker v.2.2.3 (Van Oosterhout et al., 2004). Errors due to stuttering or allelic drop-out were not detected, but null alleles were found across loci at low frequency. Null alleles were corrected using the Brookfield method (Brookfield, 1996; Van Oosterhout et al., 2004) and used for the genetic analyses. Data with corrected null alleles and uncorrected null alleles provided similar results.

3.3.2.2. Genetic diversity estimates
GenAlEx 6.5 software (Peakall and Smouse, 2012) was used for basic statistics of genetic diversity for each locus and population. In order to evaluate the degree of polymorphism for each locus the number of alleles (Na), the effective number of alleles (Ne), the number of private alleles (Ap) and the Shannon's allelic diversity index (I) to quantify the effectiveness of each locus in discriminating populations, as well as Observed (Ho) and expected heterozygositities (He).
per locus.

To evaluate genetic diversity in each population descriptive statistics were calculated using GenAlEx 6.5 (Peakall and Smouse, 2012): the mean observed number of alleles (Na), the mean effective number of alleles (Ne), the mean number of private alleles (Nap), observed (Ho) and expected (He) heterozigosities, mean inbreeding coefficient (Fis) within each population. Additionally, two estimates of the number of alleles expected in samples of specified size, the allelic richness (Ar) and the private allelic richness (Arp), were calculated with HPRare software, using the rarefaction method, in order to be able to compare populations with different sample sizes. GENEPOP v.4.2 (Raymond and Rousset, 1995) was employed to detect deviations from Hardy-Weinberg equilibrium and linkage disequilibrium across loci and populations with exact probability tests (Raymond and Rousset, 1995). Both tests used a Markov chain (1000 dememorisation steps, 100 batches, 1000 iterations/batch).

3.3.2.3. Genetic structure and differentiation

- Model-based approaches

Genetic structure was investigated using STRUCTURE v2.3.4. (Pritchard et al., 2000) which applies the Markov Chain Monte Carlo (MCMC) algorithm. This procedure clusters individuals into populations and estimates the proportion of membership in each population for each individual, without priori information on the origin of sampled individuals, in order to obtain k clusters of populations maximizing the differences between clusters while minimizing differences within clusters. An admixture model with correlated allele frequencies was used, the K value was set from one to 58, and five runs were performed for each value of K. The length of the burn-in period was set to 50,000, and the MCMC chains after burn-in were run for an additional 100,000 times. The optimal value of K was determined by examination of the ΔK statistic (Evanno et al., 2005) using Structure Harvester (Earl & Von Holdt, 2012).

Within the Bayesian clustering framework, GENELAND (Guillot et al., 2005) was proposed as improvements of STRUCTURE by integrating geographic information to infer the number of populations and detect the genetic discontinuities among these populations. GENELAND attempts to detect genetic boundaries, considering that these boundaries separate K random mating subpopulations. The main drawback of GENELAND algorithm is that it does not consider admixture in populations. In models without admixture, the sample is assumed to consist of K genetically divergent groups of individuals, and the analysis uses the genetic data to classify each individual/population in a sample into one specific group; they are not robust to
fusion events between populations. As with mitochondrial DNA we performed GENELAND analyses, with the spatial model that incorporates geographical coordinates of sampled individuals, consisted of 10 independent runs for each of the possible modelling combinations, each consisting of 100 000 iterations with a thinning of 100 after a burn-in of 50 000 iterations. MCMC convergence was assessed by comparing the number of populations across replicate runs, with the mean posterior density used as a criterion to choose the best run under a given set of model conditions. A main issue with these methods resides in the clustering approach itself: assigning individuals to groups may be a likely inappropriate strategy when individuals are genetically structured as a cline. A last approach would be to use a Mantel correlogram to detect spatial structuring in allelic frequencies across river basin scales.

- **F statistics and Analysis of Molecular Variance (AMOVA)**

As formulated, Wright’s \( F_{st} \) ranges from 0.0 to 1.0, where 0.0 indicates identical allele frequencies in a pair of populations (no differentiation) and 1.0 indicates alternate fixation for a single unique allele in each population. Extending beyond Wright’s (1951, 1965) original intent of estimating the degree of fixation, \( F_{st} \) and its analogues (fixation indexes) are often employed in empirical studies to identify, quantify, and compare the magnitude of genetic differentiation among population samples. In this context, a minimum value of 0.0 is interpreted as identical allelic composition, but a maximum value of 1.0 is interpreted as the absence of any shared alleles, and intermediate values as the magnitude of genetic differentiation among samples. If any population sample has more than one allele, however, even when the samples share no alleles in common, the maximum value of 1.0 is never observed for fixation indexes (Hedrick, 1999; Jost, 2008). Further, as the internal genetic diversity of population samples increases, the maximum possible value decreases. Consequently, using fixation indexes will systematically underestimate genetic differentiation, especially when using highly polymorphic markers such as microsatellites (Hedrick, 1999). The deficiencies of these indexes have been increasingly realized in recent years, leading to some new measures being proposed, as standardized fixation indexes, such as Hedrick’s G’st (Hedrick, 2005) and Jost’s D (Jost, 2008).

Hedrick (2005) proposed that standardizing GST relative to mean within population heterozygosity (i.e., genetic diversity) yields an improved (0,1)–scaled estimate of genetic differentiation (G’st) calculated by scaling the observed (GST) relative to its maximum achievable value (GST max), given the observed within-population heterozygosity. When population samples are completely differentiated (i.e., have no shared alleles), both Hedrick’s and Jost's standardized fixation indexes reach the maximum of 1.0, regardless of
allelic variation within populations, providing useful (scaled) measures of genetic differentiation. A comparison of different indexes can generate useful insights into the evolutionary processes that influence population differentiation (Leng and Zhang, 2011).

As for mtDNA data, the genetic variation existing between and within populations was analysed by F-statistics. In addition to $F_{st}$, Jost’s $D$ index was used as a measure of genetic distance between populations and calculated for each population pair using the web based resource SMOGDv.1.2.5 (Crawford, 2010). $D$ is a relative measure of differentiation, which ranges from zero (no differentiation) to one (complete differentiation), and simulations have shown that it is an unbiased estimator of differentiation, and outperforms $F_{st}$, over a range of sample sizes and for markers with different numbers of alleles (including highly variable microsatellite loci) (Gerlach et al., 2010). $D$ was used to test for a correlation between geographic and genetic distances and to graphically represent pairwise population differentiation in the Multidimensional scaling analysis, coupled with $F_{st}$. AMOVA was conducted using Arlequin version 3.5 (Excoffier and Lischer, 2010), with 9999 permutations to test for significance. First, AMOVA without a priori group assignment was used to determine the proportion of genetic variation partitioned among and within samples. Hierarchical AMOVA was conducted on three different grouping schemes, to determine the proportion of genetic variation partitioned among these groups and determine which data partition explains better the overall observed genetic diversity: populations were clustered into river basins (AMOVA 2), into STRUCTURE detected clusters (AMOVA 3) and finally into clusters identified by GENELAND (AMOVA 4).

• **Mantel test (IBD)**

To test for the presence of recent gene flow between populations we used a Mantel test (Mantel 1967; Smouse et al. 1986) to calculate the correlation between genetic and geographic distance for populations within the three main regional basins (Isonzo, Livenza and Tagliamento), separately. Geographic distances between sampling points were calculated on riverine distances through If gene flow does not exist between populations, we expect the correlation between genetic and geographic distance to be strongly positive, whereas if gene flow is consistent, then we expect the correlation to be near zero. We calculated statistical significance for the correlation using 9999 random permutation of the data, under the null hypothesis of no IBD, as implemented in R package Adegenet 1.4-0 (Jombart, 2008)

• **Exploratory approach: Multi Dimensional Scaling**

An alternative tool for exploring genetic data is offered by reduced space ordination methods because their utilization is not contingent on a particular genetic model. Hardy–Weinberg
equilibrium or linkage equilibrium are thus no longer required.Basically, these methods aim at summarizing strongly multivariate data into a few uncorrelated components, forming the so-called ‘reduced space’ (Corander et al., 2008). For this summary to be meaningful, the components are chosen so as to reflect most of the variability in data, as defined by an optimized criterion (for example, variance among observations). Such methods can be applied on allelic frequency data to obtain a summary of the genetic variability among individuals or populations. Multivariate methods (e.g. PCA, PcoA, MDS) represent genetic distances using a small number of synthetic variables.

Nonmetric multidimensional scaling (MDS, also NMDS and NMS) is an ordination technique that differs in several ways from nearly all other ordination methods. First, MDS is not an eigenvalue-eigenvector technique like principal components analysis or correspondence analysis that ordinates the data such that axis 1 explains the greatest amount of variance, axis 2 explains the next greatest amount of variance, and so on. In MDS, a small number of axes are explicitly chosen prior to the analysis and the data are fitted to those dimensions; there are no hidden axes of variation. Secondly, unlike other ordination methods, MDS makes few assumptions about the nature of the data. For example, principal components analysis assumes linear relationships and reciprocal averaging assumes modal relationships. MDS makes neither of these assumptions, so is well suited for a wide variety of data. MDS also allows the use of any distance measure of the samples, unlike other methods which specify particular measures, such as covariance or correlation in PCA. The non-metric MDS algorithm is an iterative procedure, constructing the MDS plot by successively refining the positions of the points until they satisfy, as closely as possible the similarity relationships between samples (Clarke and Warwick, 1994). The fit of the data in two dimensions is measured by the stress factor. MDS makes minimal assumptions about the distribution of the data and has a greater ability to represent more complex relationships accurately in low dimensional space than other multivariate methods, in our case a high number of pairwise comparisons. To visualize these pairwise relationships in a synthetic yet comprehensive way we applied non metric-multidimensional scaling (MDS) analysis to the pairwise matrix of $F_{st}$ and $D$ values through the Adegenet R package (Jombart, 2008). We used 1000 bootstrap replicates and the harmonic mean of $D$ across loci.
4. Results

4.1. mtDNA

4.1.1. Sequence data analysis

After the manual editing of sequences, we obtained 429 sequences (427 bp long) for the 16S rDNA and 449 (310 bp long) COI sequences for the whole FVG region; additionally we obtained 25 sequences from the Trentino and Lombardia samples, that were used only for the phylogenetic analysis. Sequences were aligned by Muscle algorithm (Edgar, 2004), incorporated in MEGA 5.05 (Tamura et al., 2011). Only high quality sequences were retained for successive analysis: the amplification and sequencing success rate for COI and 16S sequences was 92.5% and 89% respectively. For the COI sequences the correctness of the alignment was verified at the amino acid level.

The 16S sequence alignment comprised 497 sequences with 96 polymorphic sites, 68 were parsimony informative, and revealed 32 haplotypes in FVG, 31 of which newly described. In the Lombardia and Trentino samples 6 haplotypes were found, 4 of which were undescribed.

The COI sequence alignment comprised 497 sequences with 86 polymorphic sites, 56 of which were parsimony informative, and revealed 36 different haplotypes in FVG, 30 of which are newly described, while in the Lombardia and Trentino samples we detected 8 haplotypes, 7 of which are newly described.

4.1.2. Phylogenetic analysis

• 16S rDNA

The 16S rDNA phylogeny was inferred to assign the populations of this study to the different subspecies previously identified for the A. pallipes species complex (Fratini et al., 2005; Granjean et al., 2000). The Bayesian analysis resulted in a tree topology with the 2 presumed species clearly divided: A. pallipes and A. italicus. Within the A. italicus species two main phyllogroups, highly supported by posterior probability values, were identified; group I, which contains sequences assigned to A. i. carinthiacus and A. i. italicus subspecies, and group II including sequences of A.i. carsicus and a highly supported sister group represented by three newly described haplotypes from Lombardia and Trentino samples. The rest of the tree topology is best described as an unresolved politomy of a clade containing the 31 undescribed haplotypes found in FVG and the small number of A.i.merdionalis haplotypes available from genbank, three
from southern Italy and one from Slovenia. The 16S phylogeny shows that the *Austropotamobius* populations in FVG belong to two different subspecies of *A. italicus* species (sensu Fratini): *A. i. carsicus*, found exclusively in one site (Rosandra river), with two haplotypes, one inedited (hap 32) and one previously described (hap 10) in same site by Fratini (Fratini et al., 2005), and presumably *A. i. meridionalis* in all other regional sampling sites, with 30 haplotypes, all previously undescribed.

Fig. 5. 16S phylogenetic tree of haplotypes obtained from this study (branches in red) and downloaded from genebank; Numbers on nodes are percentage values obtained from bayesian posterior probabilities; numbers after haplotype names indicate the number of individuals sharing the haplotype. Putative species/subspecies are reported (sensu Fratini 2005). The new haplogroup found in present study, named TL, is indicated.
Fig. 6. COI phylogenetic tree. Numbers above nodes indicate posterior probability values of a given topology greater than 50%. The red branches represent haplotypes sampled in the present study. Numbers after haplotype names indicate the number of individuals sharing the haplotype. Haplotypes are assigned to the COI haplogroups proposed by Trontelj (2005). COI haplotypes of individuals belonging to the 16S TL group are indicated.
• COI

The COI phylogeny was inferred in order to validate the 16S rDNA results and to compare our data with more recent studies (Trontelj et al., 2005), that have already analyzed samples from the FVG region and nearby areas (south eastern alps and western Balkans).

Besides the subdivision between *A. pallipes* and *A. italicus* species, the tree topology supports the same main haplogroups identified by Trontelj, corresponding namely to: NW Italy, Istra 1, SEA-W Balkans, Appenine and Istra 2. In FVG 29 out of the 30 haplotypes found cluster within the SEA-W Balkans group (corresponding to individuals assigned to meridionalis subspecies by the 16S rDNA) and two haplotypes (hap23 and hap 36), found in the Rosandra river sample, cluster within the Istra 1 group, which seem to correspond to the carsicus subspecies. Hap 36 is newly described, while hap 23 has been previously described by Trontelj in the Kerso island.

The Trentino samples, assigned to the *carinthiacus* group for the 16S, belong to the NW Italy haplogroup, with three haplotypes (1 previously described and 2 undescribed); the remaining individuals from Trentino and the whole Lombardia samples display 5 peculiar undiscribed haplotypes, (hap 37-39-40-41-44) that don’t find any collocation within the known haplogroups. In particular hap 37, 39 and 44, shared by Lombardia and Trentino, strongly cluster together and cannot be assigned either to NW Italy or Istra 1 groups; (these individuals were assigned by the 16S rDNA to the sister group of *A.i.carsicus*).

4.1.3. Phylogeographic analysis

The median joining network analysis of the whole *A. pallipes* species complex included 3 main branches/major clusters, not hierarchic but rather star-like, comprising the 6 haplogroups described by Trontelj: W Europe (first cluster), Appenine, Istra 2 and SEA-W Balkans (second cluster) and Istra 1, NW Italy (third cluster). In the presumed root of the network three undescribed haplotypes from the North-eastern Italy (Lombardia and Trentino samples) seem to have originated the Istra 1 and NW Italy lineages, as well as a third additional group of two unclassified haplotypes (group TL), recorded in the Lombardy and Trentino samples. Since these basal haplotypes were found nowhere else and predictions from coalescent theory assume that older haplotypes should be interior within the network and be characterized by a higher number of descending lineages (Crandall and Templeton, 1993; Posada and Crandall, 2001), it appears clear that Northern Italy represents an important diversification center for the entire species complex. In this area it is likely to have split into NW Europe *A. pallipes* and Southern *A. italicus*, due to the vicariant effect of the Alps.
In group 3 all haplogroups are linked to the same median vector: the SEA-W Balkans group, spread from western Slovenia to the north easternmost part of Italy and along the Eastern Adriatic coast to Montenegro, includes the majority of FVG populations sampled in this study and is represented by a high number of haplotypes, connected by one or two mutational steps. Appenine group is present in Southern Italy with a less divergent group of haplotypes, originating from Mc3 haplotype, found in the Marches region (central eastern Italy) (Cataudella et al., 2010); Istra 2, represented by 2 haplotypes, was found only in the Istria peninsula by Trontelj (2005). Since these haplotypes were found nowhere else, the diversification process of these 3 haplogroups from a common ancestor probably started in the Istria peninsula, which has already been considered as an important diversity center for the A. pallipes (Trontelj), acting like a primary glacial refugia during the Pleistocene.

Fig. 7. Most parsimonious median-joining (MJ) network for A. pallipes complex COI haplotypes. Circles represent haplotypes (in black haplotypes found in present study), red diamonds symbolize median vectors (mv) that represent hypothetical missing or unsampled ancestral haplotypes; lines length indicate mutational steps connecting haplotypes; the arrow indicate the putative position of the root of the network.

The haplotype network of the FVG populations comprised 36 haplotypes, belonging to two main haplogroups: the Istra 1 haplogroup and the SEA W Balkans group (sensu Trontelj), separated by 16 mutational steps. The Istra 1 haplogroup identified exclusively the population of the Rosandra river, including two haplotypes, the most abundant Rec4HR (n=23) and the inedited hap 36.
Within the SEA-W Balkans haplogroup internal and terminal nodes can be interpreted as old vs. recently derived haplotypes; three closely related basal haplotypes, previously described by Trontelj, were the most abundant: haplotype Moo_IT (n= 77) mainly present in Livenza basin (n= 60); Cres_Hr (n=90) in the Tagliamento basin (n=75) and Ved15_IT (n=74) , the most widespread haplotype, shared by 4 out of the 6 regional basins: Tagliamento (29), Isonzo(44), Livenza (17) and Cormor (5). 31 additional haplotypes were identified, all endemic to the hydrographic basins (private haplotypes), except three shared by different basins (hap 16, hap 19 and hap 29). In particular Soc_SI haplotype was private for the Isonzo basin (n=20), haplotype 4 of the Tagliamento (n=16). The remaining 25 were low frequency (n ≤10) satellite haplotypes, separated by one or two mutational steps from the three major haplotypes in a star-like plot.

![Fig.8. Most parsimonious median-joining (MJ) network for A. pallipes complex COI haplotypes in FVG. The size of the circles is proportional to the frequencies of the represented haplotypes and colors correspond to the different regional basins (see legend). Lines are proportional to mutational steps separating haplotypes, except in the Rosandra haplogroup where line is out of scale for graphical reasons and number of substitutions is indicated. Haplotypes designation corresponds to those from Trontelj et al, (2005).](image)
4.1.4. Genetic diversity

Genetic diversity values were calculated for each station as well as for main river basins. Detailed statistics are reported in appendix (tab. 1). Haplotype and nucleotide diversity in the overall FVG region was high (Hd= 0.865, K= 4.53), compared to previous studies of similar sampling size: Pedraza et al. (2010) found significantly lower values in 53 Spanish populations (Hd=0.121, K=0.162) while a range of different values, though lower than in present study, were obtained within A. italicus populations inhabiting Alpine and Apennine tributaries of the Po River (h=0.22–0.70). When looking into diversity estimates in each site, generally low haplotype diversity was detected, with 30 stations displaying only a single haplotype. Diversity estimates calculated for major basins revealed the highest haplotype richness values in the Tagliamento and Isonzo basins, and an average nucleotide diversity decreasing along an east-west gradient (from Isonzo K=3.37 to Tagliamento K= 1.97, to Livenza K=1.51). Nevertheless when considering private allelic richness Livenza shows the highest value (Hpr=1.55) after the Tagliamento (Hpr=1.61), followed by Rosandra (Hpr=1.34). Haplotype distribution analysis, as observed in the haplotype network, revealed a high number of private haplotypes in the different FVG river basins, ranging from 12 in the Tagliamento to 1 in the Cormor.

![Graph](image_url)

Fig.9. The graph reports values of haplotype richness (Hr), private haplotype richness (Hpr) and average number of nucleotide differences (k) for the six regional basins.
4.1.5. Genetic structure and differentiation

GENELAND was able to infer seven clusters in the FVG crayfish populations using the COI and 16S datasets combined with spatial prior information. Species with low dispersal ability such as the white-clawed crayfish, are expected to display high levels of genetic differentiation between the major hydrographic basins and likely between different river systems within basins, being strongly influenced by habitat fragmentation. Our results only partially agree with this assumption, since the 3 most represented clusters (k 1, 5, 6) obtained fail to display clear differentiation between different basins and in some cases a patchy distribution of the clusters is observed. Cluster 1 is the most geographically widespread group, mainly including populations from the eastern side of Tagliamento and western Isonzo and two distant populations from south western Livenza; cluster 5 and 6, though not exclusive, were mainly represented by the Livenza and Tagliamento populations respectively.

Fig.10. FVG map of clusters identified by GENELAND analysis. Each population is represented by a colour corresponding to one of the seven genetic groups (k), as indicated in figure caption. Major regional watersheds are displayed in the background by different colours.
The spatial continuity of k1 and k5 clusters between different drainage systems can be interpreted as a result of natural or artificial translocation events. Natural translocations may derive from differences in the paleo-hydrography of the region; during the pleistocenic period, when watershed shifts and river captures were common phenomena shaping river stream direction rearrangements. Such natural phenomena can explain the sharing of cluster 1 between Tagliamento and western Isonzo populations, and cluster 5 between Livenza and some western Tagliamento populations. On the contrary, human-mediated translocations of crayfish across the different river basins has been shown to be a common event since the Middle ages and even earlier (citaz) and may better explain the patchy distribution pattern without spatial continuity of some clusters (ie. K 1 in the westernmost Livenza populations and k 6 in two populations from central Isonzo basin). Summarizing results by river basins:

- the Rosandra sample is assigned exclusively to one cluster (k 4), in agreement with phylogenetic results that identified this population as belonging to a different subspecies (A.i. carsicus).
- Livenza is the most homogeneous basin, with the majority of populations assigned to one genetic cluster (k 5).
- Tagliamento populations are mainly assigned to cluster 1 (north-western and central eastern pops) and 6 (central Tagliamento), and to three minor clusters: k 2 and k 5 (four pops from two western-central tributaries of Tagliamento), k 5 (two pops from central western TAG), and 7 (3 pops from north eastern tributary of Tagliamento basin).
- Isonzo samples are represented by three clusters: k 3 (exclusive of eastern populations), k 6 (central pops) and k 1 (western pops) shared with Tagliamento.

Tagliamento and Isonzo display significant infra-basin differentiation and geographic structure, highlighting that the current genetic structure of crayfish populations is affected by processes such as isolation by landscape fragmentation, associated with habitat alteration due to increased anthropization. Fragmentation reduces the availability of natural habitat by breaking it into discrete patches surrounded by a matrix made uninhabitable or passable by anthropogenic processes (Wilcox & Murphy 1985; Bennett 1999). This can result in drastic restrictions of previously wider distribution ranges, as could be the case of populations grouped in cluster 2, and in the loss of gene flow, further enhancing isolation and genetic differentiation.

- **AMOVA**

AMOVA results, with and without hierarchical groupings, are summarized in table 3.
AMOVA 1, conducted without a priori group assignment, points out that the observed genetic variability is mainly due to genetic differentiation between populations \((F_{st} = 0.9)\). When populations were clustered within main basins a significant part of the variance was attributed to differentiation between these groups (around 55 \%) with a still significant differentiation between populations within basins \((F_{sc} = 0.81)\). AMOVA 3 with populations grouped into the 7 clusters identified by GENELAND resulted in the greatest percentage of variation explained by the differentiation between groups (80\%), minimizing the differences among populations within groups \((F_{sc} = 0.55)\). In all analysis intra-population diversity accounted only to a minor extent to the overall genetic variability (ranging from 8-11\%).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>AMOVA-no grouping</th>
<th>Hierarchical AMOVA</th>
<th>AMOVA 3-GENELAND k</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(F_{st})</td>
<td>% total variation</td>
<td>(F_{st})</td>
</tr>
<tr>
<td>within-populations</td>
<td>9.98</td>
<td>90.02</td>
<td>8.36</td>
</tr>
<tr>
<td>among-populations (within-groups )</td>
<td>90.02</td>
<td>54.52</td>
<td>11.00</td>
</tr>
<tr>
<td>among-groups</td>
<td>(F_{st} = 0.900^{***})</td>
<td>(F_{sc} = 0.816^{***})</td>
<td>(F_{ct} = 0.545^{***})</td>
</tr>
</tbody>
</table>

Tab.3. AMOVA table and corresponding values of fixation indexes are reported:

- \(F_{st}\): differences among all populations
- \(F_{sc}\): differences among populations within groups
- \(F_{ct}\): difference among groups

P values: NS not significant; * \(P < 0.05\); ** \(P < 0.001\); *** \(P < 0.0001\).

We can state that:

- There is significant differentiation on the geographical scale, both between populations of different basins as well as between populations of the same basin, indicating a significant degree of isolation and genetic fragmentation of populations;
- populations display low levels of intra-population variability;
- clusters identified by GENELAND are strongly supported by AMOVA results the, and seem better suited than basin-based grouping to explain genetic diversity distribution on the regional scale.
4.2. Microsatellites

4.2.1. Genetic diversity

The six microsatellite markers tested were polymorphic, with a total of 119 alleles identified. The six loci showed different levels of polymorphism (fig.12), both in terms of number of alleles (from 9 in Ait11 to 39 in AP3) and number of private alleles (from 1 in Ait11 to 14 in AP3). The AP3 was both the most polymorphic and informative locus in population discrimination (I=0.816), whereas Ait11 was the least variable and less informative marker, with only one private allele and the lowest Shannon information index (I=0.455). Mean values of observed heterozigosities over all samples were lower than expected under HW equilibrium at each locus, especially at locus Ait5, Ait9 and AP2 (fig. 14). Detailed statistics about allele frequencies for each locus at each population are given in Appendix (tab 4).

![Fig.11. Diversity statistics for each locus. number of alleles (Na), number of private alleles (Nap), expected (He) and observed (Ho) heterozigosities are indicated as mean values across all populations.](image)

Detailed summary of the genetic diversity found in the studied populations is reported in appendix (tab.4). The mean allelic richness, calculated only for populations with at least 3 individuals (rarefaction sample size=5), ranged from 1.23 in population 54 (Tagliamento) to 2.99 in population 47 (Tagliamento), with an average values of 2.11. Private alleles were detected in 19 populations at different frequencies ranging from 1% to 50% . Allelic richness and private
allelic richness were also calculated for basins, with rarefaction sample size=10 (fig 13). Levels of allelic richness were similar for the three main basins: the highest value was displayed by the Isonzo basin (≈4.85), followed by Livenza (≈4.81) and Tagliamento (≈4.61). Cormor and Rosandra showed similar levels of allelic richness, while the lowest value was found in the Stella basin. Private allelic richness reached highest value (≈0.68) in the Rosandra sample, while the ranking for the other basin was the same as for allelic richness.

Fig.13. The graph reports allelic richness (Ar, in blue) and private haplotype richness (Apr, in red) values for the six regional basins.

Fig.14. Graph of mean hexpected (He) versus mean observed heterozigosity (Ho) in FVG populations.
Most populations examined were not at Hardy-Weinberg equilibrium with inbreeding coefficients (Fis) positive and significantly different from zero (after 1000 permutations) in 30 out of 58 populations, belonging to Isonzo (12 populations), Livenza basins (9 populations), and Tagliamento (pop 8). Cormor, Stella and Rosandra populations exhibited significant HW disequilibrium. The exact test for all loci and all populations revealed a significant heterozygote deficiency in these populations, as shown in figure 14. The inbreeding coefficient was negative (with a higher number of heterozygotes than expected) in four populations from Livenza (pop. 27, 30, 36, 41) and one from Tagliamento (pop. 52); tests performed to detect excess of heterozygosis were not significant. No significant linkage disequilibrium was detected between pairs of loci.

4.2.2. Genetic structure and differentiation

- STRUCTURE

![Fig.15. Results from STRUCTURE analysis under the admixture and correlated allele frequencies model. Plots show the mean log probability of the data (ln Pr(X|K)) over 10 runs for each value of K from 4 to 8 and the DK statistic (Evanno et al. 2005). Each vertical bar in the colored histograms represents the posterior probability (Q) that an individual is from a given cluster. Each colour represents a cluster as by fig caption.]

Structure supported the presence of differentiation among the populations, and the DK-method suggested five clusters as the most likely population structure (fig.15). The proportion of membership (Q coefficient) of each population to each of the 5 genetic clusters is reported in
The results show a single very distinct group for the *A.i. carsicus* Rosandra population and 3 genetic groupings that correspond to main river basins: Isonzo (k1), Livenza (k2) and Tagliamento (k5). A further cluster (k4) within the Tagliamento identified two western-central populations (Morius and partially Comugna). This latter group was also detected by mtDNA cluster analysis with GENELAND, suggesting a strong pattern of isolation of these populations, both in historical (mtDNA) and recent times (microsatellites). Though genetic structure seemed to find correspondence with main regional basins, diffused admixture was revealed between basins, especially in the Isonzo basin with introgression from the Tagliamento gene pool; few populations from Tagliamento display introgression from Livenza gene pool. Such a pattern confirms results from mtDNA cluster analysis, where a relatively diffuse genetic grouping (K1, fig. Y) were shared between populations of different basins, likely as a result of crayfish translocations. Northern Tagliamento populations (pop. 52-56-58) were not differentiated in a separate cluster as were with mtDNA analysis, suggesting ancient differentiation (mtDNA). This could reflect pleistocene isolation, when the Fella river flowed into the Danubio drainage system. The faster evolving microsatellite markers reveal more recent gene flow between these populations and the southern Tagliamento populations, successive to the spartiaque shift of the Fella into the Adriatic drainage system.

**GENELAND**

GENELAND was employed to complement the analyses run in STRUCTURE and to add a more explicit geographic component to the tests. Using the spatial model, assuming noadmixture and correlated allele frequencies, the highest mean posterior density was obtained for $K = 21$ (five replicates) identifying a significantly greater number of clusters than STRUCTURE (twenty one versus five). In figure 16 the GENELAND output is represented similar to the structure output for visual comparison.

![Fig.16. Results from GENELAND analyses under a model of no-admixture and correlated allele frequencies. The modal number of clusters was $K = 21$ (five replicates). Each cluster is represented by a colour and populations assigned to a given cluster are indicated by their code numbers below the histogram.](image)
The results show once more that Rosandra population are highly differentiated and clustered separately, as well as Morius and Comugna populations (western Tagliamento). All remaining clusters mainly show not only a strong separation between different basins as well as a significant differentiation within basins, Isonzo and Livenza basins were represented by seven genetic clusters each, while Tagliamento populations were grouped into four different groups.

the evident discordance between Structure and GENELAND results can be partially explained by differences in the algorithm and priors employed.

- GENELAND may be more sensitive to find weak clusters in space. In models without admixture, the allele frequencies are assumed to be constant over space within each cluster. Consequently, in the presence of clines of admixed populations, the sample may be partitioned into geographic regions where the allele frequencies stay approximately constant, assigning the admixed population to a new cluster, overestimating the final number of clusters.

- In GENELAND neighbouring individuals are more likely to be co-assigned to a cluster than individuals far apart. In addition, the correlation between cluster labels decreases with the distance between sampling sites, as expected under spatially restricted dispersal (Kimura and Weiss, 1964). However, although the Markov property accounts for local dependencies, the model does not allow us to estimate the magnitude and scale of spatial correlations in presence of patterns of isolation-by-distance. Like many species with limited dispersal, the genetic population structure of A. pallipes is likely to be characterized by a pattern of IBD (i.e. a positive correlation between genetic and geographical distances). The analysis of data from natural populations usually involves uncontrolled factors and simplifying model assumptions, and IBD can be a confounding factor for population genetic inference (Guillot et al., 2009; Meirmans, 2012). In particular, IBD may cause the number of clusters (K) to be overestimated when using clustering methods, regardless of the inclusion of spatial information as a prior (Frantz et al., 2009; Guillot et al., 2009; Schwartz and McKelvey, 2009)

- **Mantel test (IBD)**

Isolation by distance (IBD) between populations within main river basins was tested to better understand GENELAND results. Genetic divergence conformed significantly to an isolation-by-distance pattern for the Isonzo and Livenza basin populations, corroborating the GENELAND overestimation of genetic groupings. On the contrary tests were not significant for the Tagliamento populations.
Given that IBD can be a natural feature in a species with low dispersal ability, the lack of such a pattern can indicate different scenarios: one in which populations are clustered into sharply divided groups due to natural barriers or human-mediated habitat fragmentation, and one in which populations are continuously distributed across the landscape and have greater dispersal potential with gene flow homogenizing allele frequencies. In the case of Tagliamento basin both scenarios may be merged: when habitat conditions allow dispersal there is gene flow within the spatially close populations, but also significant barriers exists that sharply divide groups, even in spatially close populations, as pops 47 e 50 from pop 55.

• AMOVA

The AMOVA without a priori groups reveals that around 60% of the observed variance is due to intra-population diversity with a significant amount of variation to be attributed to population differentiation ($F_{st}$ = 0.4).

Hierarchical AMOVA was conducted grouping populations into: basins (2), structure clusters (3) and finally GENELAND clusters (4). Disregarding the a priori groupings, results indicate that intra-population diversity explains most of the variability observed, with levels ranging from 57% to 59% of the total variance (r $^2$ = 0.38, P = 0.101).

| Source of variation | AMOVA-no grouping | Hierarchical AMOVA | AMOVA 1 | AMOVA 3 | AMOVA 4-
<table>
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<tr>
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<tbody>
<tr>
<td></td>
<td>F statistics</td>
<td>% total variation</td>
<td>F statistics</td>
<td>% total variation</td>
<td>F statistics</td>
</tr>
<tr>
<td>within-populations</td>
<td>/</td>
<td>59.78</td>
<td>$Fst$=0.429***</td>
<td>57.07</td>
<td>$Fst$=0.437***</td>
</tr>
<tr>
<td>among-populations (within-groups )</td>
<td>$Fst$= 0.402***</td>
<td>40.22</td>
<td>$Fsc$=0.314***</td>
<td>26.22</td>
<td>$Fsc$=0.268***</td>
</tr>
<tr>
<td>among-groups</td>
<td>/</td>
<td>/</td>
<td>$Fct$=0.167***</td>
<td>16.71</td>
<td>$Fct$=0.230***</td>
</tr>
</tbody>
</table>

Fig. 17. AMOVA table and corresponding values of fixation indexes are reported for AMOVA without a priori grouping (AMOVA 1) and for three differnet hierarchical clusters: populations were pooled into Basins (AMOVA 2), into Structure clusters (AMOVA 3) and into GENELAND clusters (AMOVA 4). $Fst$: differences among all populations; $Fsc$: differences among populations within groups; $Fct$: difference among groups. P values: NS not significant; * P < 0.05; ** P < 0.001; *** P < 0.0001.

The first hierarchical AMOVA, testing for differences between major basins, showed that $Fct$ was significant, indicating that 16.7 % of variance could be attributed to differences between drainages and around 20% to differences between populations within basins. For the second hierarchal AMOVA, a higher and significant portion of variance was explained by differences among groups (23%) identified by STRUCTURE, decreasing the variance between populations within groups (20.7%). Variance between groups is maximized when populations are clustered...
into the GENELAND groups, explaining up to 28% of total variance, while variance between pops within groups is minimized (13%).

- **Multi Dimensional Scaling**

MDS analysis was conducted on two different pairwise differentiation matrices, $F_{st}$ and $D$, and results are reported in fig. X and Y.

Fig. 17. MDS plots of $F_{st}$ (above) and $D$ (below) pairwise comparisons between populations.
Overall population differentiation appeared better represented by the pairwise D matrix, obtaining a final stress value of 0.040 indicating a good-excellent fit of the distances in the graph to the original genetic distances, with a correlation value \((r=0.89)\). MDS conducted on pairwise \(F_{st}\) values displayed a final stress value of 0.048 and a high correlation value \((r=0.79)\) between original distance matrix and MDS generated distances. In both analysis populations were plotted mainly across dimension 1 (D1), and show a certain degree of differentiation between main river basins. Nevertheless in the \(F_{st}\) MDS river basins displayed a weaker clustering pattern than in D MDS analysis, being basically squeezed in a central cloud.

A common pattern observed in both MDSs is the widespread position of Isonzo populations, which displayed the greatest intra-basin differentiation: three main groups were detected, corresponding to eastern, central and western populations. Following this geographical gradient Isonzo populations get closer to the Tagliamento populations. In both analysis Livenza populations form a quite compact group, clustering closer to Eastern Isonzo populations in the \(D\) MDS, approximating to Tagliamento and Rosandra samples in the \(F_{st}\) MDS. While Central Tagliamento populations were always grouped close together, a few outlier populations display varying degrees of differentiation: Eastern Tag pops approximates the Livenza and central Isonzo group; Morius (pop. 50) displays a high degree of differentiation from all pops, as previously outlined by Structure and GENELAND analysis. Cormor and Stella are placed in the proximity of Tagliamento samples.

The main difference between the two MDS analysis is represented by the position of the Rosandra sample, lacking a clear differentiation in \(F_{st}\) MDS, in contrast with previous genetic structure and phylogenetic analysis, while in the D MDS it appears clearly separated from the rest of regional populations. The discordance observed between the two MDS plots can be explained by the intrinsic differences in the two indexes used: \(F_{st}\) and \(D_{jost}\). \(D\) differs from \(F_{st}\) in a fundamental definitional way: \(D\) indicates the proportion of allelic diversity that lies among populations and is more related to the genetic distance between populations than to the variance in allele frequencies, like \(F_{st}\); as a result the two indexes can behave quite differently.

\(F_{st}\) is often interpreted as a measure of differentiation between subpopulations, whereas it actually measures the fixation of alleles. \(F_{st}\) necessarily approaches zero when gene diversity is high, even if subpopulations are completely differentiated. Indeed as the mutation rate of neutral markers increases, \(F_{st}\) decreases: a higher within-population heterozygosity yields a lower \(F_{st}\) value (Alcaide et al., 2008). In its capacity as a measure of population differentiation Jost’s \(D\) reaches its maximum value of 1 when populations do not share alleles even when there remains variation within populations, avoiding any impact of within-population diversity on estimates of
genetic differentiation among populations (Jost, 2008). Moreover F–statistics and analogues are based on the Gini-Simpson index of diversity (Gini, 1918; Simpson, 1949), a second order diversity measure, that deliberately weights common alleles more heavily than rare alleles (Jost, 2007, 2008), as likely happened in the case of the Rosandra sample, resulting in a lower genetic distance from the other regional populations; D on the contrary gives more weight to the private alleles present in the Rosandra sample, increasing its genetic distance from other samples.
5. Discussion

5.1. Taxonomic status and phylogeography of the *A. pallipes* complex

The divergence among the main lineages of *A. italicus* seems to be older than the Pleistocene and their current distribution ranges probably reflect allopatric divergence and glacial survival in separate refugia from where different post-glacial colonization routes were established. Molecular data from different animal and plant species confirm southern Mediterranean peninsulas of Iberia, Italy and the Balkans as major ice age refugia, and in most cases demonstrate that genetically distinct taxa emerged from them (Hewitt, 1996, 1999, 2004). Our results from phylogenetic and phylogeographical mtDNA analysis identified two main centers of lineage diversification for the species *complex*: Istria peninsula and North-Eastern Italy. Istria region has been considered an important ice age refugium for different freshwater species (Klobučar et al., 2013; Trontelj et al., 2005; Verovnik et al., 2005) and, according to Trontelj et al. (2005), it represents the speciation centre of *Austropotamobius* into ‘western’ *pallipes* and ‘eastern’ *torrentium* and their centre of radiation, given the presence of highly differentiated and basal mitochondrial haplotypes in a relatively small geographical area.

The 16S phylogeny revealed that the highest genetic diversity observed so far for the *A.i. Meridionalis* subspecies is present in the FVG region rather than in Southern Italy (SI), where this subspecies was first described (Fratini et al., 2005). Moreover no haplotypes were shared between FVG and SI populations, excluding the human translocation hypothesis, that had been suggested by Fratini to explain the presence of an *A.meridionalis* haplotype in a Slovenian sample. Results from COI phylogeny and haplotype network confirm that three *A.italicus* lineages, SEA-W Balkans, Appenine and Istra 2 (likely corresponding to *A.i. meridionalis* subspecies), are monophyletic and likely derived from a common ancestor in the Istria region.

Three major routes of dispersal originating in the Istria region can be inferred: the first towards the south western Balkan Peninsula, the second towards north-east (Slovenia, FVG) and the third towards southwest to the Apennine peninsula. Range expansion towards Southern Italy likely took place during Pleistocene glacial oscillations, with a north-south colonization route. In fact during the Pleistocene glacial periods the Mediterranean sea level was 100-200 m lower than its present level. This implies that the shallow northern part of Adriatic Sea, most likely dried out and therefore the Po and FVG river basins, along with the river basins from the opposite Istrian and Dalmatian coast could have come in contact. Crayfish may have dispersed through river confluence from the Istra peninsula to central Italy when the river Po delta flowed into the
Adriatic sea at the Marches region height, originating the Appenine COI group and further spreading to Southern Italy. In agreement, the COI network revealed that all haplotypes belonging to the Appenine group derive in a star-like plot from an haplotype sampled in the Marches region, where a north-to-south colonization scenario for the *meridionalis* subspecies has been suggested by a previous study (Cataudella et al., 2010) on the basis of genetic diversity distribution patterns. The dispersal of crayfish from the Marches region to SI on both sides of the Appenines could be explained either by natural dispersion or artificial translocations. In the natural dispersion scenario crayfish could have crossed the Appenine ridge using river captures or inversion of flow direction between valleys joining opposite sides of mountain ranges, as reported for other freshwater species in the Alps and Dinarides (Bianco 1995). Human translocations have played an important role in shaping the crayfish distribution across Europe (Reynolds and Souty-Grosset, 2011), and may better explain the low diversity of Southern Italy crayfish populations revealed from previous studies (De Vico et al., 2011; Fratini et al., 2005), as originating from few source populations and possibly closely related individuals. More extensive genetic studies on southern Italy populations would be needed to validate any assumption.

It has already been pointed out that Northwestern Italy might have played a particularly important role in shaping phylogeographical patterns in the white-clawed crayfish, as it has a very high genetic (and morphological) variation (Grandjean et al., 2002; Largiadèr et al., 2000), with a suture zone in the Apennine and Alpine Po affluents where the distribution of the two species of the *complex*, A.pallipes and *A italicus*, overlaps. The presence of different lineages in a small geographic scale in Northern Italy has been identified with a mixed pattern of secondary contact of relict populations associated with the Alpine region, probably related to past glacial microrefugia (Pedraza-Lara et al., 2010; Trontelj et al., 2005).

A further hot spot area for crayfish diversity was identified in this study around the Garda lake, between Trentino and Lombardy regions, which show both high lineage and nucleotide diversity, in spite of the low sample size. In the Chiese basin an undescribed group of haplotypes was detected, not fitting in any previously described COI clusters. The position of three of these haplotypes next to the COI network root suggests that Northern Italy is probably the origin of diversification of the NW Italy and Istra 1 lineages, as well as of a third newly described haplogroup, named TL (Fig. 7), that represents a highly supported sister group of the *carsicus* subspecies with the 16S gene phylogeny (Fig. 4).

So far it is not possible to assess with confidence where the species *complex* has originated, but the geographic distribution of the different lineages within the *A italicus* species seems to suggest an allopatric divergence of the *A.i italicus* species in two major areas: Istria (for the SEA-W
Balkans and Appenine COI groups, namely *meridionalis* 16S subspecies) and Northern Italy (for the NW Italy and Istra 1 COI groups, namely *carinthiacus*, *italicus* and *carsicus* 16S subspecies). Our findings suggests that the presence of unsampled lineages and eventually subspecies cannot be excluded, highlighting the need for a taxonomic revision of the entire species. Since present day taxonomy and nomenclature are only partially describing the complex biogeographical distribution and evolutionary history of the different lineages.

5.1.1. Phylogeography and evolutionary history of FVG populations

The presence of *A. i. carsicus* subspecies in FVG can be explain by two hypotesis: The first hypothesis is based on the natural distribution of the *carsicus* subspecies in the whole FVG region in pre-Pleistocene, as its distribution in the nearby Veneto region could suggest, congruently with the existence of a north eastern Italy biogeographic district. The palaeoclimatic record of the Middle Pleistocene in the Friulian-Venetian Plain (Fontana et al., 2010) suggests that the maximum glacial advance could be referred to a cold period of the Middle Pleistocene (Muttoni et al., 2003) when the ice cover reached the Friulian plain south of Udine up to 200 meters above sea acted as a glacial micro-refugial area, for the extant A.i. carsicus populations. Previous studies on freshwater decapod species displayed evidence that Karstic habitats provided refuge for different aquatic animals during glaciations (Sket, 1999; Verovnik et al., 2005). During the colder periods of the Pleistocene, probably only populations living in or near thermal refugia (e.g., underground water bodies) avoided extirpation. After the retreat of the ice a colonization of the *meridionalis* subspecies (SEA-W Balkans lineage) from Istrian refugia might have taken place with a range expansion towards the north eastern mountains of Slovenia and FVG, allowing the establishment of new crayfish populations in the habitats left empty by previous glaciation events. Given that populations containing greater genetic variation are usually located more closely to the centre of diversification or refugia from where they spread, the high haplotype and nucleotide diversity observed in the Isonzo basin is in accordance with the proposed natural colonization scenario of *A.i. Meridionalis* subspecies from the Istria peninsula into the FVG, following an east to west route. However we cannot exclude the possibility that *A.i. carsicus* immigrated into the karstic Levantine basin towards the end of the ice ages from Istria refugia and that on the immigration path crayfish experienced strong bottlenecks which reduced their genetic diversity. The second hypothesis considers an anthropogenic origin of the *carsicus/istra1* population in the Rosandra basin. Artificial translocations of crayfish have been documented in Europe since the
Middle Ages until recently (Cukerzis, 1988) and may have significantly affected the current distribution of the A. pallipes complex.

Low mitochondrial genetic variation in this carsicus population can be interpreted as evidence of a glacial relict population undergone a severe bottleneck during the ice ages, or as evidence of a founder effect due to human mediated transport from a source population located in the nearby Istrian area. The most abundant COI haplotype has been previously recorded by Trontelj in the Rijeka area (north-eastern Istra).

Further elucidation of the phylogeographic history of this A.i. carsicus population requires more genetic data both from the Rosandra stream and from neighbouring watercourses of the Levantine basin, where no crayfish populations where found in the present monitoring and from southern Slovenia for comparison. Additional data from different molecular markers, such as microsatellites, are needed in order to discriminate between the two alternative evolutionary hypotheses described above.

5.1.2. Proposal for the taxonomic revision of the A. pallipes complex

Our proposal is that an updated taxonomy should take into account:

- the identification of lineages (subspecies) reflecting consensus taxa between mtDNA markers (16S and COI), combined with more variable genetic markers, such as microsatellites.
- present day distribution combined with knowledge of past phylogeographical patterns for the different lineages (subspecies);

In order to assess an overall correspondence between 16S and COI phylogeny, some points can be highlighted:

- the COI haplogroup defined North Western Italy includes both CR (carinthiacus) and I (italicus) lineages, considered distinct sub-species by the 16S mtDNA (Fratini et al., 2005; Grandjean et al., 2000), as previously pointed out by Chiesa et al (2011).
- Carsicus subspecies (16S) seems to partially correspond to the Istra 1 (COI) phylogroup, because newly described sub-clade TL is assigned to the carsicus ssp as its sister group in 16S tree phylogeny, while no supported correspondence is found either with the Istra 1 COI group, or with any other group.
- Meridionalis subspecies includes SEA-W Balkans and Appenine COI haplogroups; a
moderate subdivision (PP=0.68) between Southern Italy and FVG samples is observed also with 16S topology. Istra 2 is likely to be grouped within *meridionalis* spp, being the sister group of Appenine taxon in the COI phylogeny.

*A.i. meridionalis* (which means “Southerner”) was the established name by Fratini (Fratini et al., 2005) for some specimen from four central-southern Italian drainages (Duranna, Nera, S. Antonio and Coscile). Our results bring evidence that this subspecies has a wider distribution range than previously thought, including the easternmost part of northern Italy (FVG) as well as Slovenia and the Blakans. This evidence coupled with the likely origin of radiation of the group in the Istria peninsula suggests that the name should be revised and that a suitable name for this clade could be *A.i. adriaticus*.

5.2. Genetic diversity and population health status

One of the main goals of present study was to investigate genetic variability of FVG white-clawed crayfish populations in order to infer their health status, since genetic diversity represents the raw material for evolution and adaptation. More genetic diversity in a species or population means a greater ability for some of the individuals in it to adapt to changes in the environment and thus to the long term survival of the species/populations.

Given that the extant genetic diversity of a species reflects the influence of both historical and recent evolutionary events, a double approach was applied to perform this task, using both types of molecular markers (mtDNA and microsatellites). On one hand mtDNA sequence variation has proven to be a useful tool in defining taxonomic position and major phylogeographical patterns within species, due to its unique attributes, (maternal inheritance, no recombination, higher mutation rates than most nuclear genes), including the *A. pallipes* species-complex. On the other hand microsatellite nuclear loci, for their higher mutation rates, tend to recover genetic variability faster after the action of processes that affect it. In this way they represent useful tools to address questions related to current population structure of species, including the white-clawed crayfish.

Results for COI mtDNA analysis revealed overall high levels of genetic variability in the FVG region, with abundant and diversified haplotypes found, mostly inhetited. This amount of mtDNA diversity suggests a long evolutionary history of white-clawed crayfish populations in the FVG region, with divergence of basal haplotypes likely dating back to the Pleistocenic colonization of FVG regional watersheds by the *A.i.meridionalis/SEA-W Balkans* lineage from the Istra peninsula. Nevertheless when the distribution of genetic variance is considered, we
detect low levels of within population diversity that account barely to the 10% of the total regional diversity; most of the variability is explained by significant differences among populations ($Fst= 0.90$), both within and between river basins ($Fct= 0.54$), consistent with the presence of many population/basin-specific private haplotypes. Higher levels of intra-population genetic variability were detected by microsatellite markers, as expected by their faster mutational rate. Moreover, mtDNA has a haploid genome and is usually maternally inherited, resulting in an effective population size that is $1/4$ of that for nuclear DNA, so that genetic drift is more pronounced and fixation rates faster: haplotypes can be wiped out during bottleneck events, reducing faster within-population genetic diversity.

Although extensive studies with SSR markers on crayfish species are still scarce, relatively low genetic variation appears to be characteristic of white-clawed crayfish populations. Nevertheless I found moderate levels of genetic variation of $A. pallipes$ in FVG populations with microsatellite markers, with an average expected heterozigosity (dependant on the number of alleles sampled) of 0.416, considerably higher than that found in previous studies involving populations across France ($H=0.235$) (Gouin et al., 2006), and in populations from Tyrol ($H=0.301$) and Carinthia ($H=0.350$) (Baric et al., 2006, 2005). Recently Matallanas reported comparable levels of heterozigosities in several populations throughout Spain ($H= 0.420$). In terms of observed heterozigosity ($Ho=0.276$) the FVG populations show levels of genetic variability similar to or slightly higher than those described for other European populations examined with SSR markers ($Ho=0.186$, Matallanas et al., 2013; $Ho=0.110$, Bertocchi et al., 2008; $Ho=0.220$, Gouin et al., 2006), with the exception of South Tyrol populations ($Ho=0.53$, Baric et al., 2005).

It must be said that comparisons with other studies are to be taken with caution, since the use of different SSR loci can result in substantial differences in the genetic diversity estimates; while loci Ap2 and Ap3 have been applied in previous studies (Gouin et al., 2006; 2002; Matallanas et al., 2013; Baric et al., 2005), loci Ait 1, 5, 9 and 11 have only been tested in few individuals from Spanish and tuscanian populations, but never applied to an extensive population study before present.

The general heterozigosity deficiency observed in most samples across SSR loci are likely the result of reduced effective population sizes. Indeed the rate of inbreeding, a frequent phenomena in small population, was relevant in many populations (see appendix tab.4), although these results must be interpreted with caution, since a limited number of specimen sampled decreases the chance of sampling all possible genotypes at the different loci, as may be the case of many populations in present study.
The genetic variation of white-clawed crayfish in FVG has been and likely still is affected by successive bottleneck events, and consequently by the effect of genetic drift; indeed it has been demonstrated that white-clawed crayfish populations can experience drastic reductions in size during severe river droughts or floods, periodic pollution events (fertilizers, pesticides) and disease break outs.

5.3. Population structure and differentiation

According to the classical and spatial analysis of genetic differentiation performed, significant levels of population differentiation were observed both at the COI analysis ($F_{st} = 0.91$) and SSR analysis ($F_{st} = 0.402$). Hierarchical AMOVA reveals that a significant portion of the total diversity is ascribable to differentiation between river basins ($F_{ct} = 0.167$) and the comparison between $F_{st}$ and D indexes in MDS analysis gave a similar spatial clustering pattern allowing a discrimination of main basins, except for the Rosandra sample positioning (see results chapter); these results are congruent with STRUCTURE analysis, that identified five different gene pools characteristic of main regional basins. The Genland results were incongruent with Structure concerning the identification of genetic clusters, estimating a surprisingly high number of clusters ($K = 21$). This indicates that structure is more conservative and other studies suggested that spatial clustering models may provide more accurate estimates of K over nonspatial models when genetic differentiation is low, which is not the case in FVG crayfish populations. Two main factors may be responsible for such incongruence: isolation by distance (IBD) of populations and the presence of admixed populations.

IBD can be a confounding factor for population genetic inference (Guillot et al. 2009; Meirmans 2012). In particular, IBD may cause the number of clusters ($K$) to be overestimated when using clustering methods including spatial information as a prior (Frantz et al. 2009; Guillot et al. 2009; Schwartz & McKelvey 2009). The presence of clines of admixed populations, the sample may be partitioned into geographic regions where the allele frequencies stay approximately constant, assigning the admixed population to a new cluster, overestimating the final number of clusters.

These results highlight the importance of comparing results from multiple programmes because clustering algorithms are heavily influenced by model choice and priors (Guillot et al. 2009). Nevertheless, besides these technical considerations, the discordance of clustering analysis underlies some meaningful biological issues that can help infer population genetic structure, and eventually be used for planning management strategies:
1. Do Populations display an isolation by distance pattern and do they suffer from habitat fragmentation?
2. Is there detectable admixture between river basins and is this as a result of human mediated translocations?

5.3.1. Habitat fragmentation

As observed in other studies on freshwater crustacean species (Gervasio et al., 2004), the populations of the main river basins displayed signs of differentiation according to a model of isolation by distance, a pattern expected for neighbouring populations in a species with limited dispersal ability (Clarke, 2000). This pattern is generally enhanced by the increasing fragmentation of freshwater ecosystems through habitat degradation and pollution, resulting in small isolated populations that are more vulnerable to environmental changes and demographic fluctuations, increasing the risk of local population extinction.

These results bear interesting implications for the management of crayfish populations: if possible, connections among streams should be established to promote gene flow among populations, in order to increase their genetic variability and ensure their long term stability.

5.3.2. Admixture: natural or artificial translocations?

Genetic admixture occurs when individuals from previously separated populations begin interbreeding. Admixture results in the introduction of new genetic lineages into a population. It has been known to slow local adaptation by introducing foreign, unadapted genotypes (known as gene swamping). It also prevents speciation by homogenizing populations. Genetic admixture often occurs when a geographic barrier separating populations, such as a river, is removed or when anthropogenic activities result in movement of populations.

Spatial analysis with the coupled mtDNA markers revealed the presence of trans-basin genetic clustering, suggesting a contact between population from different drainage systems. Since regional basin have been isolated for thousands of years and mtDNA can bear the molecular footprint of ancient biogeographical patterns, this phenomenon could be interpreted as the result of ancient colonization events. Natural translocations may indeed derive from differences in the paleo-hydrography of the region; during the pleistocene period, when watershed shifts, river captures and stream direction rearrangements were likely responsible for the spread of crayfishes into the region. This is congruent with the COI haplotype network (fig. 8) where the most
represented hplotype (VED15_IT) is shared between different basins, likely representing the basal haplotype that successively diverged into basin-characteristic haplotypes and further on into single river-private haplotypes. Nevertheless, given the intrinsic characteristics of mtDNA it is not possible to distinguish between historical and recent admixture events nor infer the direction of the introgression process.

SSR loci, given their high mutational rates, carry the molecular footprint of more recent admixture events affecting current population structure. Moreover SSR markers are able to assess individual admixture levels, because of its bi-parental inheritance. The STRUCTURE cluster analysis revealed strong admixture in the Isonzo populations and it was possible to detect the direction of the introgression from the Tagliamento gene pool (fig. 15).

This results seem to contrast the hypothesis of natural colonization driven by expansion from Isonzo basin into Tagliamento drainage system. Nevertheless the two scenarios are not mutually exclusive since recent artificial crayfish translocations from Tagliamento to Isonzo may over shade natural historical movements, through the homogenization of allele frequencies.

Admixture was also detected in Eastern Tagliamento populations with introgression from Livenza gene pool, while Livenza basin displayed introgression from Isonzo basin in few western-central populations. The admixture observed through SSR analysis is likely to be the result of artificial secondary contact between crayfish populations from different basin.

Uncontrolled translocations of crayfish can have a significant impact on the genetic differentiation of populations due to the genetic “pollution” of original gene pools with the consequent loss of potential adaptation to the local environment.

5.3.3. Identification of ESUs and Mus

An important goal in conservation genetics is to characterize evolutionary lineages within endangered species before making management decisions. Our data from microsatellite markers, consistent with mtDNA results, brings evidence of strong historical population differentiation between FVG southernmost populations of A. pallipes (Rosandra stream) and the rest of the region, as previously discussed. I propose that these two groups of populations should be considered as two Evolutionary Significant Units (ESUs), representing two separate conservation units in management programs. The significant differentiation revealed by clustering analysis between basin suggests that populations could be managed by river basins in conservation plans. Indeed river basins represent an obvious management target in freshwater species because they represent a structure in which gene flow has been confined for thousands of years. Such an
approach would reduce the risks of mixing genes from different pools, that may be adapted to different environments, eroding the natural differentiation that characterizes FVG native crayfish populations.

5.4. Management and conservation implications

The decline of white-clawed crayfish populations observed during the RARITY monitoring program in FVG has led to growing concern and management strategies have recently been implemented in order to endorse the preservation and stability of extant populations. A main issue in conservation strategy planning is the restocking and reintroduction of individuals to increase long-term population stability and/or restore previously locally extinct populations. The activities of captive breeding and reintroduction can be divided into several phases. The first is represented by traditional biological monitoring coupled with genetic monitoring (preliminary to every conservation action) in order to evaluate the status of wild populations including estimations of population size, genetic variability and population structure. Genetics may play an important role in the identification of populations of conservation priority, through the detection of genetic diversity declines leading to high inbreeding rates and eventually to decrease fitness. The second step is the selection of breeders from natural populations for the establishment of a broodstock for the ex-situ reproductive practices and the production of offspring to be used for restocking/ reintroduction into the wild. In this phase the contribution of genetic data can be very useful in the choice of suitable individuals for the constitution of a broodstock. Indeed, For the enhancement of wild stocks it is important to create a broodstock containing in the genome most of the genetic diversity found in natural populations, whilst minimizing the risk of genetic pollution in the releasing sites through the introduction of genetically incompatible individuals. The genetic data obtained in present study enable us to express some recommendations for future management practices of native crayfish, with particular emphasis on restocking and reintroduction activities. To summarize the main issues, it would be recommended:

• To consider the two ESUs identified as separate management and conservation units, with separate local broodstocks for eventual restocking actions;
• Main river basins should be preferably considered as different management units, though the genetic mapping produced suggests that some populations from different basins could be managed as a unique group, given their genetic similarity, for example western Isonzo populations could be restocked with individuals coming from adjacent Tagliamento
populations, given their genetic homogeneity, rather than with individuals belonging to eastern Isonzo populations, which display high genetic differentiation.

- Within management units it is possible to increase genetic diversity of the populations to be reinforced inter-crossing breeders from different neighbouring localities and if possible, connections among streams should be established to promote gene flow among populations, in order to increase their genetic variability and ensure their long term stability.
6. Conclusions

In conclusion, this study provided new insights into the biogeography and taxonomic status of the *A. pallipes complex* in Friuli Venezia Giulia. Our results underscore the utility of employing both mitochondrial and nuclear loci to evaluate levels of genetic variability and current structure of white-clawed crayfish populations. Genetic information of present study should be taken into account for future conservation plans, such as restocking and reintroduction, including the monitoring of priority populations.
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