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Population dynamics and biological connectivity among Italian smooth newt (*Lissotriton vulgaris meridionalis*) populations inhabiting a network of wetlands in a traditional landscape

Dinamiche di popolazione e connettività biologica tra popolazioni di tritone punteggiato italiano (*Lissotriton vulgaris meridionalis*) che abitano un network di aree umide in un paesaggio tradizionale

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PREFACE

This Ph.D. thesis is focused on the population dynamics and biological connectivity of the Italian smooth newt in a traditional landscape. The thesis is structured as follow:

GENERAL INTRODUCTION. This section briefly addresses the issues related to the conservation value of artificial aquatic habitats for amphibians. The introduction is structured in three subsections, in accordance with the following three chapters. In the first subsection, I explore the ecological value of drinking troughs for amphibian conservation. In the second subsection, I highlight the special need for the application of conservation genetics to amphibian populations, and for the development of suitable molecular tools. In the third subsection, I examine the conservation genetic value of drinking troughs for amphibians. Finally, in each subsection I enunciate the specific aims of the Ph.D. research project.

CHAPTER 1. The chapter is conform to the submitted paper: "Buono V, Bissattini AM, Vignoli L. Can a cow save a newt? The role of cattle drinking troughs in amphibian conservation. Acta Oecologica".

CHAPTER 2. The chapter is conform to the accepted paper: "Buono V, Galliani G, Mancini E, Davoli F, Mengoni C, Mucci N, Vignoli L. An improved microsatellite panel to assess genetic variability in the Italian smooth newt (*Lissotriton vulgaris meridionalis*). Journal of Genetics".

CHAPTER 3. The chapter is based on the submitted paper (abstract not included): "Buono V, Bissattini AM, Davoli F, Mengoni C, Mucci N, Mancini E, Vignoli L. Fine-scale spatial genetic structure and dispersal among Italian smooth newt (*Lissotriton vulgaris meridionalis*) breeding populations in a rural landscape. Molecular Ecology".

FINAL CONCLUSIONS. The conclusion section briefly highlights and discusses the main outcomes of the present thesis in the light of the proposed aims.

SUMMARY

Dinamiche di popolazione e connettività biologica tra popolazioni di tritone punteggiato italiano (*Lissotriton vulgaris meridionalis*) che abitano un network di aree umide in un paesaggio tradizionale

Un crescente numero di studi suggerisce che i fattori antropogenici, più che le fluttuazioni naturali, siano responsabili del declino delle popolazioni di anfibi in tutto il mondo. Per gli anfibi dell’area mediterranea, la perdita, l’alterazione e la frammentazione degli habitat, legati all’intensificazione dell’agricoltura e dello sviluppo urbano, rappresentano le principali minacce al loro declino. In particolare, gli habitat acquatici, che rappresentano i siti riproduttivi d’elezione per la maggior parte degli anfibi, richiedono una profonda attenzione a causa della loro allarmante riduzione in termini di quantità e qualità. Recentemente, è emersa l’importanza degli habitat acquatici artificiali come surrogato per la riproduzione, la colonizzazione e il sostentamento di popolazioni vitali di molte specie di anfibi. È stato osservato che anche i siti acquatici artificiali di minori dimensioni (es. fontanili, cisterne e serbatoi), associati ad attività agricole e di allevamento di tipo tradizionale, giocano un ruolo fondamentale in questo fenomeno. Tuttavia, pochi studi in letteratura si sono focalizzati su tali sistemi, a favore di quelli di grandi dimensioni (laghetti e pozze), costruiti con caratteristiche che richiamano quelle dei siti naturali. In questo contesto, i fontanili vengono facilmente colonizzati dagli anfibi, svolgendo una funzione complementare ai corpi idrici naturali, in ambienti profondamente antropizzati. Ad oggi, l’esistenza di questi sistemi artificiali è a rischio a causa della modernizzazione e intensificazione dell’agricoltura, e del conseguente abbandono delle pratiche tradizionali a basso impatto, che hanno garantito per decenni la persistenza di queste strutture. La progressiva scomparsa di questi habitat artificiali può portare alla perdita progressiva di popolazioni isolate a livello locale, come già successo in Spagna e in Italia. Per tali ragioni, l’identificazione e la protezione di questi siti chiave risulta prioritaria nell’ambito della conservazione degli anfibi a livello ecologico e genetico.

Il tritone punteggiato italiano (*Lissotriton vulgaris meridionalis*) è stato scelto come specie modello del presente progetto di ricerca in

quanto, in Italia, risulta essere minacciato dalla frammentazione e distruzione degli habitat idonei, in particolare quelli acquatici, ed è solito colonizzare con successo sistemi acquatici artificiali. Teatro del presente studio sono i Monti della Tolfa, un sito localizzato in provincia di Roma, caratterizzato da un profilo rurale interessato da pratiche agricole e di allevamento tradizionali. L'allevamento brado della vacca Maremmana è particolarmente diffuso e determina la presenza cospicua di fontanili disseminati nella matrice del paesaggio.
Il presente progetto di ricerca si propone di:

- ☐ Studiare le dinamiche di popolazione della specie nell'area di studio e identificare le caratteristiche dei fontanili che favoriscono la persistenza della specie a lungo termine
- ☐ Sviluppare un set di marcatori genetici (microsatelliti) idonei allo studio della variabilità genetica e all'identificazione della struttura di popolazione a scala fine nella specie
- ☐ Analizzare il ruolo dei fontanili nel mantenimento della diversità genetica e nell'ambito dei processi di dispersione della specie

Il primo capitolo del presente progetto di ricerca evidenzia che i fontanili con determinati tipi di caratteristiche possono contribuire alla conservazione del tritone punteggiato italiano supportando popolazioni spesso numerose e ben strutturate. In questo contesto, la vacca Maremmana sembra agire da "ingegnere ecosistemico" incrementando direttamente l'eterogeneità del paesaggio, e fornendo indirettamente habitat acquatici idonei e fondamentali per la sua riproduzione. Ciò testimonia che, anche siti acquatici artificiali di piccole dimensioni, opportunamente gestiti, possono aiutare a mantenere popolazioni vitali di anfibi in ambienti caratterizzati da scarse aree umide.

I risultati del secondo capitolo del presente progetto di ricerca testimoniano che i marcatori microsatelliti, caratterizzati precedentemente per altre specie/sottospecie del genere *Lissotriton*, rappresentano uno strumento idoneo per analisi genetiche a scala fine nel tritone punteggiato italiano. L'ottimizzazione di tale set di marcatori permetterà di approfondire le dinamiche di popolazione della specie, allo scopo di indirizzarne efficacemente le strategie di gestione e conservazione.

Nel terzo capitolo e ultimo capitolo del presente progetto di ricerca si sottolinea l'importanza dei fontanili nel mantenimento dei processi di dispersione e flusso genico tra i demi di tritone punteggiato italiano all’interno di un network costituito da siti acquatici artificiali e naturali. I fontanili si sono dimostrati in grado di supportare popolazioni ben strutturate e con un buon livello di variabilità genetica, collegate da processi di flusso genico, la cui intensità è in buona parte influenzata dalla distanza geografica che le separa. Il grado di connettività riscontrato tra sistemi acquatici naturali e artificiali suggerisce l’esistenza di una metapopolazione, in cui i processi di colonizzazione, successivi ad eventuali estinzioni locali, potrebbero essere altamente probabili.

Il presente progetto di ricerca riconosce il ruolo dei fontanili nella conservazione degli anfibi a livello ecologico e genetico, colmando un vuoto di conoscenze che può aver portato alla loro sottovalutazione nell’ambito della ricerca e delle politiche ambientali, a favore esclusivo dei sistemi acquatici naturali.

GENERAL INTRODUCTION

1) Exploring the ecological and functional value of drinking troughs for amphibian conservation

A growing number of studies suggest that anthropogenic factors more than natural fluctuations are responsible of the decline of amphibian populations all over the world (Stuart et al. 2004). Amphibians are particularly vulnerable to a wide range of detrimental factors since they require both terrestrial and aquatic habitats during their life cycle (Alford et al. 2001). For Mediterranean amphibians, the major threats are represented by habitat loss, alteration and fragmentation caused, in particular, by agricultural intensification and urban development (Stuart et al. 2004; Cushman 2006). Aquatic habitats, representing reproductive sites for many amphibian species, deserve a particular attention because their reduction in number and quality is alarming (Stevens et al. 2002). However, in recent years it has clearly emerged that artificial aquatic sites as surrogates of natural ones (e.g. man-made ponds with smooth shorelines and aquatic vegetation) represent an important contribution to the potential breeding habitats for amphibians (Petranka et al. 2003; Rannap et al. 2009; Lannoo 2014). Even small artificial aquatic sites, extremely different in shape, structure, and size in comparison to natural sites (e.g. tanks, drinking troughs and reservoirs with water surface < 10 m^2), have been identified as suitable habitats for amphibian reproduction (Romano et al. 2014). Indeed, the ability of amphibians to successfully colonize artificial water bodies has been well documented worldwide (Petranka et al. 2003; Rannap et al. 2009; Lannoo 2014). However, most of artificial habitats, especially those associated with traditional agriculture and cattle watering, are endangered by modern and intensive farming, and the abandonment of traditional irrigation practices (Garcia-Gonzalez and Garcia-Vazquez 2011). Artificial water bodies may play an important role in maintaining viable populations of amphibians, and their disappearance may cause the local loss of small isolated populations, as was the case in Spain (Casas et al. 2012) and in NW Italy (Canessa et al. 2013).

With increasing anthropogenic habitat modification it is particularly important to investigate the ecology and biology of individual species, their local population dynamics and the demographic consequences of changes in landscape composition and configuration (Semlitsch 2000; Manel et al. 2003). Indeed, our ability to improve management and conservation of amphibian species is limited by the lack of basic information on population dynamics. Demographic parameters driving changes in population size both temporally and spatially seem to be unknown at most (Cushman 2006). Several studies highlighted the importance of artificial water bodies for amphibians (Contreras et al. 2009; Knutson et al. 2004), especially in the Mediterranean (Temple and Cox 2009), but most of them focused on artificial sites that simulate natural ponds (Contreras et al. 2009; Knutson et al. 2004). Thus, assessing the role of the smaller and completely artificial sites used for agriculture and livestock in sustaining viable amphibian population is not only timely but also of special concern (Romano et al. 2014). Although preventing the modernization of agriculture is not possible and would be undesirable from a socio-economic viewpoint, the identification and protection of such key sites should be mandatory for amphibian survival (Garcia-Gonzalez and Garcia-Vazquez 2011), especially when demes are structured in metapopulations, a dynamic population model frequently observed occurring in amphibians (Sjögren 1991; Marsh and Trenham 2001). To explore the role of small artificial aquatic habitats in amphibian ecology and conservation, I selected the Italian smooth newt (*Lissotriton vulgaris meridionalis*) as a model species and I investigated the importance of drinking troughs for the conservation of the species in an area interested by traditional farming and agriculture. The Italian smooth newt is an ideal candidate for this kind of study; the species, in fact, is threatened by fragmentation and destruction of suitable habitats, especially of reproductive sites, and is known to colonize successfully ditches, cisterns and drinking troughs (Razzetti and Bernini 2006). The primary goal of the first chapter of the present Ph.D. thesis was to provide a scenario on the basic demographic attributes of Italian smooth newt populations inhabiting drinking throughs and to identify trough characteristics favoring the persistence of the species in the long term. Given the alarming fast rate in aquatic habitat loss and alteration, drinking troughs may represent the only suitable site for amphibian reproduction within an unsuitable habitat matrix by playing a significant role in favoring amphibian population persistence and recovery from pristine habitat loss. The recognition of the conservation value of traditional drinking troughs, whose importance have been neglected in research and policy in favor of natural aquatic systems, is timely, especially in light of the current major changes in local landscapes, due to land use intensification, land abandonment, and infrastructural and urban development (Romano et al. 2014).

2) Improving a tool for the conservation genetics amphibian populations

Amphibians are undergoing major global declines and in recent decades have become more threatened than birds or mammals (Stuart et al. 2004). To help stop amphibian decline and for the correct management and conservation of amphibian species, a deep knowledge of the ecology and biology of individual species, their local population dynamics and their genetic structure is urgently required (Purrenhage et al. 2009). The gene pool of most species is structured and comprised of multiple populations that vary in the rate of exchange of individuals (Newman and Squire 2001). Amphibian populations often show strong site fidelity (Sinsch 1991; Kusano et al. 1999) and are relatively isolated with a significant differentiation even at a fine scale (i.e. < 10 km) (Rowe et al. 2000; Shaffer et al. 2000). However, some species depart from this pattern and show patchy distributions (Newman and Squire 2001), due to habitat specificity and strict ecophysiological requirements (Stebbins and Cohen 1995), and are organized in regional networks of subpopulations, or metapopulations (Sjögren 1991; Marsh and Trenham 2001). Habitat fragmentation ranks among the major causes of amphibian species declines (Stuart et al. 2004), and one obvious consequence of fragmentation is the breakdown of effective individual's dispersal processes among demes (Gonzalez et al. 1998) which decreases the probability of regional population persistence (Sjögren 1991). The empirical estimation of individual exchanges among populations at the landscape scale is problematic due to logistic factor (long term studies needed are time and money consuming) (Burns et al. 2004). This highlights a special need for the application of conservation genetics to amphibian populations, and the development of suitable molecular markers (Jehle and Arntzen

2002). Genetic data have been used to investigate levels of dispersal and molecular researches have contributed to further understanding of amphibian population dynamics (Shaffer et al. 2000; Newman and Squire 2001). Neutral genetic markers, mostly microsatellites, have been used successfully in studies of amphibian effective population sizes and structures, dispersal and migration patterns (Beebee and Griffiths 2005). Amphibians are good genetic models because they are (i) widely distributed in most ecosystems; (ii) easy to sample in breeding assemblages; (iii) often showing high site fidelity for reproduction thus generating high levels of population genetic structure; (iv) amenable to controlled crossings in the laboratory; and (v) of major conservation concern (Beebee and Griffiths 2005). However, although they appear highly suitable for addressing population and conservation genetic issues, amphibians are still under-represented in this research area (Jehle and Arntzen 2002). Indeed, the cloning of microsatellite loci for amphibian species remains, compared to other groups of organisms, a time- and money-consuming endeavour (Steinfartz et al. 2004) because of their large genome sizes and low numbers of potentially amplifiable loci (Hendrix et al. 2010; Hauswaldt et al. 2012). Being aware of this problem, several studies (Krupa et al. 2002; Primmer and Merila 2002; Zhan and Fu 2008) tested the cross-amplification suitability of species-specific amphibian microsatellite loci in closely related congeneric species (Krupa et al. 2002; Garner et al. 2003). Similar approaches have been also successfully applied to other vertebrate groups such as birds (Primmer et al. 1996) and pinnipeds (Hoffman et al. 2007). However, in amphibians, the results often showed unexpected low rates of amplification success probably because of their large genome sizes (Garner 2002), and/or the taxonomy that is inconsistent with phylogenetic relationships among taxa and therefore obscures the existing genetic divergence between taxa (Primmer and Merila 2002).

With increasing anthropogenic habitat modification it is particularly important to understand the genetic consequences of changes in landscape composition and configuration, even at a small geographic scale (Johanet et al. 2009). Thus, I selected the Italian smooth newt (*Lissotriton vulgaris meridionalis*) as a model species and I identified a suitable tool for addressing conservation genetic issues for this potentially threatened species. Differently to other European newts, in fact, *Lissotriton vulgaris meridionalis* is not protected by national law in our country, although local populations seem to be experiencing severe declines due to habitat fragmentation and loss (Razzetti and Bernini 2006). The primary goal of the second chapter of the present Ph.D. thesis was to test and optimize a panel of microsatellite markers, previously developed in other *Lissotriton* species and subspecies (*L. montandoni, L. vulgaris graecus, L. helveticus*, and *L. vulgaris*), on the Italian smooth newt in order to assess population structure and genetic variability at a local spatial scale. Indeed, microsatellites represent the suitable markers for investigating regional population connectivity of the species because it (i) has limited dispersal capabilities, (ii) exhibits high breeding site fidelity, and (iii) is a highly deme-structured species (Griffiths 1996; Roth and Jehle 2016). For the Italian smooth newt, no microsatellite loci have been reported so far and their development will be an important contribution to better understand the ecology and evolution of the species, and its population genetic structure and variability across many levels of scale, from metapopulation to full geographical range. Such microsatellite markers will consequently assist proper management decisions and future conservation of this potentially threatened species.

3) Exploring the conservation genetic value of drinking troughs for amphibians

Amphibians are considered the most threatened vertebrates under the current conditions of climate change and human modifications of their natural habitats (Stuart et al. 2004). Amphibian species' declines are topical and well publicized (Alford and Richards 1999) but are still little studied from a genetic point of view (but see Shaffer et al. 2000), although the amount of genetic variation could affect fitness-related traits (Rowe et al. 1999). Amphibian populations generally differ from the panmictic population model (Kalezić and Tucić 1984). Indeed, they often have patchy distributions, due to habitat specificity and strict ecophysiological requirements (Stebbins and Cohen 1995), show low dispersal abilities and are philopatric, leading to distinct populations that can represent unique genetic entities despite geographic proximity (Scribner et al. 2001). Most amphibians depend on both aquatic and terrestrial habitats, and, for protection and management plans, their local population dynamics as well as the degree of population connectivity must be assessed by considering a wide suite of habitat types (Semlitsch 2000). This is particularly true for pond-breeding amphibians that attend specific and distinct environments for courtship behavior, breeding, larval development and adult survival (Pope et al. 2000). Most amphibian breeding sites consist in naturally isolated patches (i.e. pools, ponds, lakes). Therefore, those amphibian species inhabiting such breeding sites are naturally organized in fragmented ranges with isolated populations. The comprehension of dispersal processes represents, therefore, a critical factor for population dynamics and sustainability in order to assist landscape management and conservation planning (Hanski 1998; Semlitsch 2000). When connectivity is disrupted, in fact, genetic and demographic rescue of threatened subpopulations may become impossible (Purrenhage et al. 2009). However, our knowledge about actual dispersal patterns and the geographical scale at which gene flow is limited in amphibians is deficient (Zamudio and Wieczorek 2007). Although population size fluctuations can be directly addressed with standard field methods in medium- and long-term studies, the empirical assessment of the exchange rates of individuals among populations could be very problematic (Burns et al. 2004). Many studies suggested that direct observations of individual movement through mark-recapture methods are informative (Driscoll 1997; Dodd and Cade 1998) but are inefficient at detecting dispersal rates over long periods (decades), long-distance dispersal and at demonstrating if dispersal has resulted in effective breeding (Burns et al. 2004). Conversely, microsatellite markers have proved to be sensitive indicators of change in dispersal rates (Scribner et al. 1994). Moreover, these markers are deemed suitable tools for addressing questions related to mating systems (Jones et al. 2002; Jehle et al. 2007) and population subdivision and interconnection even at fine-scale (Jehle et al. 2005; Johanet et al. 2009).

With increasing anthropogenic habitat modification it is particularly important to understand the genetic consequences of changes in landscape composition (Guerry and Hunter 2002; Manel et al. 2003). Fragmentation of contiguous habitat by agriculture and urban development can create barriers to amphibian dispersal (Vos et al. 2001; Johansson et al. 2005) resulting in isolated subpopulations more subject to increased risk of local extinction (Frankham 2005; O'Grady et al. 2006), and/or less likely to receive dispersers from neighboring subpopulations (Purrenhage et al. 2009). However, landscape features (i.e. vernal pools, drainage, ditches, and streams) and artificial facilities (e.g. tanks, drinking troughs and reservoirs) may act as stopovers during dispersal events, thereby facilitating amphibians' movements by reducing effective inter-aquatic systems distances (Gibbs 1998; Pope et al. 2000). The importance of artificial water bodies, generally associated with traditional agriculture and cattle watering, for amphibians feeding and reproduction has been highlighted by many studies in the Mediterranean (Romano et al. 2014). However, there are no studies on their potential stepping-stone function in the dispersal process and for the gene flow maintenance. Thus, I investigated the impact of drinking troughs on amphibian population connectivity within a patchy system interested by infrastructural and agricultural development by focusing on the Italian smooth newt (*Lissotriton vulgaris meridionalis*) as a model species. The selected species is abundant and widely distributed in Latium (Central Italy) (Razzetti and Bernini 2006), and represents a good focal species for examining regional population connectivity. Similarly, drinking troughs represent an ideal model for investigating deme organization and dynamics in aquatic habitats (Jeffries 2005). The primary goal of the third chapter of the present Ph.D. thesis was to determine if drinking troughs support viable amphibian breeding populations and if and how they are connected to other natural and/or artificial aquatic systems. Studies that clarify the dynamics of demes are important to understand amphibian population ecology and are also particularly useful in applied ecology (Purrenhage et al. 2009). Specifically, studies that estimate the spatial scale of dispersal among subpopulations, in addition to basic demographic research, are needed in order to provide data for effective management and conservation decisions (Purrenhage et al. 2009). The determination of genetic relationships among populations could assist management practices, including identifying areas of high conservation priority, revealing dispersal corridors among populations, detecting severe population declines and identifying individuals and/or populations fundamental for augmentation, translocation or reintroduction programmes (Mace et al. 1996).

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CHAPTER 1

Can a cow save a newt? The role of cattle drinking troughs in amphibian conservation.

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Abstract

Amphibians are the vertebrate group experiencing the steeper global population decline and species loss. Habitat alteration and loss caused by the intensification of agriculture are among the main causes of their decline. However, in the historic past amphibians have been favored by traditional agriculture since more terrestrial and aquatic habitats became available through the diversification in land use and the creation of man-made facilities for field irrigation and cattle watering. It has been suggested that drinking troughs for cattle watering may represent the only permanent water points for amphibian reproduction and sheltering in human modified landscapes. Here, we focused on *Lissotriton vulgaris meridionalis*, a sub-endemic newt, widespread in Italy, whose populations seem to face a fast decline due to habitat loss and fragmentation. The primary goal of this study was to determine the importance of drinking troughs for the ecology and conservation of the species in a hilly area (Latium, Central Italy) interested by livestock farming (i.e., Maremmana cow), agricultural cultivation and urban development. Specifically, we analysed, using Generalized Linear Models (GLMs), the effects of drinking trough characteristics (i.e. size, substrate, aquatic vegetation, intensity of the management) and landscape features (i.e. distance to the forest, forest cover, percentage of cultivated lands and infrastructural development, terrestrial connectivity) influencing the occurrence and abundance of the species. In the light of our results, we can conclude that drinking troughs sharing specific characteristics represent suitable alternatives to pristine habitats for *L. vulgaris meridionalis*. In this context, the presence of Maremmana cow indirectly provides suitable breeding sites crucial for the conservation of the species. This demonstrates that even small and man-made aquatic habitats, associated with traditional husbandry practices, may help to sustain amphibian populations in landscapes where natural wetlands are scarce. Our study is valuable since it fills a gap of knowledge arising from the lack of empirical data on amphibian ecology in small artificial habitats that may have been neglected in research and policy.

Introduction

Amphibians are the vertebrate group experiencing the steeper global population decline and species loss (Stuart et al., 2004). Many amphibian species require both terrestrial and aquatic habitats during their life cycle, which makes them particularly vulnerable to a large range of detrimental factors acting on and across both environments (Alford et al., 2001). Habitat alteration, introduction of alien species, over-exploitation, global change and infectious diseases are considered the main causes of amphibian decline (Stuart et al., 2004).

Currently, nearly 45% of the European Union is covered by farmlands whose agricultural practices have been shown to greatly impact amphibian populations (Joly et al., 2001). Indeed, the intensification of agricultural practices has resulted in field size enlargements, urban extensions and an increase in monoculture reducing the diversity and availability of amphibian terrestrial habitats (Arntzen and Teunis, 1993). In areas under continuous agricultural use there has been an increase in the arable to pasture ratio, with a concomitant decline in numbers of suitable aquatic sites for amphibian reproduction and development (Oldham and Nicholson, 1986; Dudgeon et al., 2006). It has been estimated that wetlands have increasingly disappeared over the last seven decades in southern Europe (up to 61% in Spain and 64% in Italy; CEE, 1995; Casas et al., 2011). However, in the historic past, amphibian populations have been favored by extensive agricultural practices since more terrestrial habitats and breeding sites became available through the diversification in land use and the creation of man-made facilities for field irrigation (ponds) and cattle watering (tanks, reservoirs and drinking troughs) (Arntzen and Teunis, 1993; Knutson et al., 2004; Hartel et al., 2014; Romano et al., 2014). It has been suggested that in many areas troughs for cattle watering may represent the only permanent water points for amphibian reproduction and sheltering in human modified landscapes (Knutson et al., 2004; Garcia-Gonzalez and Garcia-Vazquez, 2011; Hartel et al., 2014). Drinking troughs are shallow (20-60 cm depth) man-made habitats, often in connection with natural permanent aquatic systems, with elongated rectangular shape and vertical walls that amphibians easily climb to reach the water (Denoël, 2004). The ability of amphibians to colonize successfully artificial aquatic sites, in fact, is a worldwide phenomenon (Petranka et al., 2003; Rannap et al., 2009; Lannoo, 2014). However, most studies have focused on artificial sites simulating natural ponds (e.g. man-made ponds with smooth shorelines and aquatic vegetation) (Contreras et al., 2009; Knutson et al., 2004), whereas those extremely different in shape and size in comparison to natural sites (e.g. tanks, drinking troughs and reservoirs with water surface < 10 m^2) remain almost neglected (Romano et al., 2014). To date, the persistence of such artificial systems is endangered by land reclamation, urbanisation and the abandonment of traditional farm practices in favor of modern agriculture and intensive farming (Romano et al., 2014). The disappearance of such artificial aquatic habitats may cause the local loss of small isolated amphibian populations, as was the case in Spain (Casas et al., 2012) and NW Italy (Canessa et al., 2013).

Complex life-cycle organisms like amphibians represent good models to study the consequences and adaptations to the environmental changes at the aquatic-terrestrial interface that are expected to occur in the forthcoming decades because of agricultural intensification and global changes (Miaud and Merilä, 2001). In this study, we focused on the Italian smooth newt (*Lissotriton vulgaris meridionalis*), a small, semiaquatic salamander widespread in Italy (Razzetti and Bernini, 2006). The species completes the biological cycle in a wide variety of lentic and lotic freshwater habitats, both natural and artificial, and in the surrounding terrestrial habitats from sea level up to 1700 m (Razzetti and Bernini, 2006). Although accurate data on the density and abundance of this species are overall still lacking, *L. vulgaris meridionalis* populations seem to face a fast decline due to habitat loss and fragmentation (Razzetti and Bernini, 2006). Given this concern, the aim of the present study was to provide data on the basic demographic attributes and population sizes of the Italian smooth newt in a hilly area in the northern part of the Latium region (Central Italy) interested by livestock farming, agricultural cultivation and urban development. Moreover, we aimed at determining the importance of drinking troughs for the ecology and conservation of the species in human modified environments. Specifically, we analyzed the effects of several factors (abiotic and biotic) that potentially influence the occurrence and abundance of the species, including landscape features and drinking trough characteristics.

A deep understanding of demography and habitat requirements of the Italian smooth newt during the aquatic and terrestrial phase is a prerequisite to assess its conservation status and to plan management actions in farmland landscapes (Denoël and Lehmann, 2006). Moreover, our study is valuable because it attempts to recognize the role of drinking troughs for amphibian conservation filling a gap of knowledge arising from the lack of empirical data on amphibian ecology in such small artificial habitats (but see Garcia-Gonzalez and Garcia-Vazquez, 2011) that may have been neglected in research and policy in favor of lakes, rivers and streams (Fuentes-Rodrìguez et al., 2013).

Materials and Methods

Study site

Fieldwork was carried out in the Tolfa Mountains (elevations between 150 m and 450 m a.s.l.), a hilly region situated approximately 60 km north of Rome (Latium, Central Italy). The study site falls in the Maremma district, extended throughout Latium and Tuscany, and includes a large established Natura 2000 site belonging to the hypomesaxeric subregion of type B (Tomaselli et al., 1973). The study site, 70.000 ha in surface, is characterized by a Mediterranean-temperate climate with cold winter (without snow covering), rainy spring and autumn, and dry and hot summer (Tomaselli et al., 1973). Vegetation mainly consists of riverine woods (*Ulmus campestris*, *Salix* sp., *Populus* sp.), wide areas covered by bushy pastures (*Spartiurn junceum*, *Cytisus scoparius*, *Prunus spinosa*, *Rubus ulmifolius*, *Crataegus monogyna*) and mesophilous forests (*Quercus cerris*, *Q. pubescens*, *Ostrya carpinifolia*) (Spada, 1977).

The study site has a biodiversity value among the highest in Italy despite the proximity to several main cities. It shows a rural profile interested by traditional husbandry and agriculture, with medium to low urban and infrastructural development, where troughs for livestock watering (cattle, horses and donkeys) are particularly common and widespread. The most bred species is the Maremmana cow, a robust native breed of cattle frugal and well-adapted to difficult environments. The Maremmana cow was particularly exploited between the two world wars and became close to extinction because of the increasing mechanization of agriculture and draining of marshes. To date, there are around 20.000 animals in three regions—Marche, Lazio and Tuscany—with the largest farms found in Maremma. The fact that the Maremmana cow cannot be kept indoors and ranges in the wild reveals its perfect adaptation to the harsh terrain and poor pastures of the Maremma, where it is capable to survive in a semi-feral state with a minimum management effort. In this context, the Maremmana cow may be considered as an ecosystem engineer since it modulates directly or indirectly the availability of spatial resources to other species, by causing physical state changes in biotic materials (Jones et al., 1994). Indeed, it maintains open habitats that otherwise would be replaced by wooded areas and provides additional aquatic resources represented by small artificial water bodies built by farmers to facilitate its management.

All these environmental and landscape features make the study area an ideal candidate for studying how traditional farming practices can sustain amphibian viable populations by providing surrogates of natural aquatic habitats.

Fieldwork

In 2015-2016 years a comprehensive survey of drinking troughs and natural permanent aquatic systems (i.e., brooks, lakes and ponds) was conducted thoroughly exploring the study site and locating them on topographical maps (Google Earth©). Newt surveys were started in the breeding season from the beginning of March until the middle of June in both sampling years. Populations inhabiting drinking troughs are assumed to be closed to demographic changes among captures carried out during the site-visit day and open to demographic changes between sampling visits. We define ‘site-visit’ any single visit to a site during which three or more surveys are conducted to assess population estimates. Thus, we assume that sites are open to changes in population size between site-visits, but are closed to changes in occupancy during site-visits. Conducting multiple surveys during each site-visit (single day) minimize the time over which closure is assumed still providing the detection and nondetection data necessary to estimate newt occurrence (cf. MacKenzie et al., 2006). As single visit within a season may lead to imperfect detection, each aquatic system was sampled at least two times by the same person for a maximum of an hour and thus minimizing habitat disturbance (MacKenzie et al., 2002). In drinking troughs, we searched for newts by dip-netting, carrying out blind sweeps from side to side in order to cover the aquatic system systematically and exhaustively (Arntzen, 2002). In natural aquatic systems we walked along the shoreline, visually located and captured newts by hand and nets. Collected newts were temporarily maintained in tanks filled with water, marked by means of a photograph of the ventral pattern and classed by sex and stage. Newts showing secondary sexual characteristics (cloacal swelling, dorsal crests in males) were considered adults, individuals lacking those characteristics were classed as juveniles, whereas individuals with three pairs of external gills and a developed cloaca were considered paedomorphs (Razzetti and Bernini, 2006; Denoël, 2017). Newts were released in their aquatic habitat after observations.

Removal sempling and Hayne's (1949) techniques were used to estimate the absolute densities of newts in drinking troughs. Removal sempling is a method of absolute density estimation based on the decline in successive catch numbers as individuals are removed from a population (Gist and Crossley, 1973). We performed at least three dip-netting sweeps to gather a minimum number of three individual collections. Newts caught in each sweeps were removed from the drinking troughs and temporarily kept in tanks filled with water until all samplings were completed. When the number of individuals caught on a given occasion is plotted against the total cumulate number caught on previous occasions, the total number in the population can be estimated by fitting a curve to the plotted points and reading the cumulative total beyond which the number of animals subsequently caught is zero (Hayne, 1949; Bell, 1977).

For each drinking trough, we recorded the presence/absence of newts (eggs, larvae, adults) and the total number of individuals collected. We also recorded the following drinking trough features: (i) size, estimated by surface area (cm^2) and volume (cm^3), (ii) type of substrate on the bottom (sedimentary-mud or rocky), (iii) percentage surface cover of floating, emerged and submerged vegetation, and (iv) the intensity of the management activities (how often a trough is cleaned and the severity of cleaning activity: i.e. from just algae removal to complete cleaning with chemicals). To evaluate the landscape features, topographical maps were analyzed to measure: (i) distance (m) to the edge of the closest forest, (ii) percentage of forest cover within a 400-m radius centred at each drinking trough, (iii) percentage of human disturbance (cultivated lands, urban and infrastructural development), within a 400-m radius centred at each drinking trough, and (iv) estimated terrestrial connectivity among adjacent aquatic systems, estimated as the number of water bodies (artificial and natural) within a 400-m radius centred at each drinking trough. We considered a 400-m radius centred at each drinking trough since this is the average migration distance of *L. vulgaris* as suggested by Joly et al. (2001).

Data analyses

We used descriptive statistics to summarize the occurrence of natural and artificial permanent aquatic systems and the percentage of occurrence of newts in the various aquatic sites. The t-student test was used to compare pooled sex-ratio between natural and artificial aquatic systems. Differences in newt body size between sexes and among aquatic systems were tested using one-way ANOVAs. Individual measures are expressed as mean±SD.

For the analyses we selected the abundance and presence of newts [presence (1) vs absence (0)], the occurrence of paedomorphic newts [presence (1) vs absence (0)], and the population abundance (population estimate by removal sampling) as dependent variables. As for the independent variables, we used Factor Analysis (FA) with principal components as extraction method to reduce the amount of environmental variables and mitigate the potential bias due to multicollinearity among measured variables. FA identifies a few orthogonal axes in multivariate distributions that summarize the majority of variation in the data set and expresses these axes as linear combinations of the original variables (Everitt and Dunn, 2001). We used the following 11 continuous, countable and discrete variables (for countable and discrete variable we indicate the codes for each considered state): (i) trough size (both surface and volume), (ii) type of substrate on the bottom [sedimentary-mud (1) vs rocky (0)], (iii) percentage of floating, emerged and submerged vegetation, (iv) the intensity of management activities [no management (0) vs moderate management with vegetation control and surface cleaning once a year (1) vs intensive management (2) resulting in periodic cleaning and disinfection with chemicals, (v) distance (m) to the edge of the closest forest, (vi) percentage of forest cover, (vii) percentage of human disturbance, and (viii) terrestrial connectivity among adjacent aquatic systems, determined by physical barriers to migration such as roads and cultivated lands [isolated (0) vs connected in one direction (1) vs connected bi-directionally with other neighboring aquatic systems (2)]. We considered as significantly correlated to a factor the variables with loading above 0.71 (Tabachnick and Fidell, 2001); we retained principal component axes with eigenvalues greater than one for further analyses. A varimax rotation was applied to the FA axes to facilitate their interpretation. We then interpreted the principal component axes by examining loading of each variable on the rotated axes. We perform a FA for the full dataset of drinking troughs (including all the 51 drinking troughs filled with water) and a further FA on the reduced dataset including only the site where newt occurred for the analyses on newt abundance and occurrence of paedomorphs.

We used the FA factors as independent continuous variables in Generalized Linear Models (GLMs). A first GLM assuming binomial error distribution (logistic link function) was performed to analyze newt presence and the occurrence of paedomorphosis (dependent variables, binomial distribution) in relation to drinking trough and environmental features. A second GLM (normal error distribution and identity link function) was used to test the effect of drinking trough chracteristics and landscape features on abundance of newts (population estimate; dependent variable, normal distribution).

Prior to analyses, continuous variables were transformed to achieve normal distribution: newt abundance, drinking trough size and the distance to the edge of the closest forest were log (x + 1) transformed, whereas percentages of vegetation, forest cover and human disturbance were arcsin transformed to linearize relationships.

All statistical tests were performed using Statistica (Statistica 8.0 for Windows) with a significance level of 0.05.

RESULTS

Body size and population size in relation to sex and habitat type

Overall, a total of 7 natural permanent aquatic systems and 58 drinking troughs were located and georeferenced in the study area. The study species occurred throughout all the natural permanent aquatic systems detected and at 27 (47%) drinking troughs. Fifty-one drinking troughs (88%) contained water and were still used for livestock, while 7 (12%) were empty, damaged or completely abandoned by landowners (Fig. 1).

Overall, we collected 259 individuals (112 males and 147 females) in natural permanent aquatic systems and 1279 individuals (561 males, 590 females and 128 paedomorphics) in drinking troughs. The sex-ratio bias was very similar in both aquatic systems and was slightly biased toward females (pooled sex-ratio 1:1.05 for drinking troughs; pooled sex-ratio 1:1.31 for natural permanent aquatic systems; t-test: $t = 0.758$; $n = 29$; $P = 0.454$). As for body size, individuals from natural permanent aquatic systems were larger than those collected in drinking troughs (SITE TYPE effect: $F_{30,\ 1379} = 19.274$, $P < 0.0001$; Fig. 2), with a clear sexual dimorphism in both aquatic systems with females larger than males (SEX effect: $F_{1,\ 1406} = 51.2$, $P < 0.0001$; SITE TYPE*SEX effect: $F_{1,\ 1406} = 0.176$, $P = 0.895$; Fig. 3). The population estimate varied from a minimum of 10-14 individuals (FPz, FGi and FPa) to a maximum of 132-214 individuals (FE and FT) per site (Tab. 1). The largest populations were observed at two drinking troughs connected to natural permanent aquatic systems and located in enclosed canopy, close to the nearest forest.

Relationships among Italian smooth newt demography, drinking trough characteristics and management

As for newt presence in drinking troughs, the FA extracted four factors with eigenvalues > 1, with 73% of total explained variance. Ten variables out of 11 showed a significant association to the FA factors (Tab. 2). GLMs revealed a significant positive relationships between the occurrence of *L. vulgaris meridionalis* in drinking troughs and factors 1 and 4 (the effect of the latter is marginally non-significant). Newt occurrence was positively associated to mud substrate of troughs, a high percentage of submerged vegetation cover, a high degree of trough connectivity (associated with factor 1, 29% of explained variance), a large cover of emergent vegetation and a low intensity of management actvities (associated with factor 4, 12% of explained variance) (Tab. 3).

As for the occurrence of paedomorphic individuals and newt population estimate, FA extracted four factors with eigenvalues > 1, with 79% of total explained variance. The association of the tested variables and the FA factors was the same estimated by the FA performed on the full dataset and are not shown for brevity reasons. GLMs revealed a significant positive effect of drinking trough size (both surface and volume) and the occurrence of paedomorphic individuals (Factor 1 effect: Wald = 4.186; $p = 0.041$). Moreover, GLMs showed a positive significant relationship among newt abundance, forest extent and distance to the edge of the closest forest (Factor 3 effect: Wald = 15.900; $p < 0.0001$).

DISCUSSION

Main findings

Our findings demonstrated that even smaller and completely artificial aquatic habitats, linked to traditional farming practices, provide irreplaceable breeding sites for amphibians and an essential contribution to their conservation. Contarily to the general expectation that free-ranging cattle have detrimental effects on amphibian occurrence in artificial ponds (Knutson et al., 2004; Braun, 2006), we provided robust evidence that the presence of extensive cattle breeding may have positive effects on amphibian ecology and survival. At our study site, drinking troughs, associated with the Maremmana cow breeding, provided suitable alternatives to pristine habitats for the reproduction, feeding and sheltering of *L. vulgaris meridionalis*. In this context, the Maremmana cow seems to act as an ecosystem engineer (i) directly by structuring landscape features (i.e., increasing habitat heterogeneity by grazing) and (ii) indirectly by providing suitable breeding sites crucial for the conservation of the Italian smooth newt. Indeed, traditional husbandry practices determine the increase of the total amount of aquatic habitats available to the Italian smooth newt, thus helping to sustain its populations in a region where natural aquatic sites are increasingly disappearing. We can conclude that even small and man-made aquatic habitats, properly managed, may help to sustain amphibian populations in landscapes where wetlands are scarce.

Body size and population size in relation to sex and habitat type

Our study provided population demographic data for the Italian smooth newt inhabiting natural and artificial permanent aquatic systems, in particular drinking troughs, a habitat never investigated in the past. In drinking troughs newt populations reached high densities (up to 55 ind/m^3) demonstrating that such simplified environments with limited spatial and trophic resources, may successfully act as surrogates of natural pristine sites for their reproduction.

In our study populations, body sizes were in the range of observed lengths in other related populations on the Tyrrhenian coast with coastal individuals smaller than those from the mainland (Nobili and Accordi, 1997; Pizzuti Piccoli, 2008). In newts, females are often larger than males (Steward, 1969; Bell, 1977), and our populations conformed to this rule. Indeed, body size is an important trait in *Lissotriton vulgaris* life-history: large males have a greater supply of sperm during the breeding season and select larger and more fecund females (Verrell and Francillon, 1986), whose eggs hatch earlier (Bell, 1977). The observed sex ratio slightly biased towards females can be possibly explained by the phenology of the study populations: our sampling period partly coincided with the end of the breeding season when males are less active or leave the water (Arntzen, 2002).

We also found that individuals from natural permanent aquatic systems were larger than those collected in drinking troughs. The observed differences in size attained at the adult stage should not be related to the aquatic habitat stability as reported by Nobili and Accordi (1997), since drinking troughs are supposed to represent stable water bodies as well as natural water systems. Newts have a complex life cycle consisting of a pre-metamorphic aquatic larval stage and a post-metamorphic juvenile and adult semi-aquatic stages (Griffiths, 1996). Adult body size is attained mainly during the juvenile phase of life, but aquatic larval phase is also thought to be responsible for adult growth (Halliday and Verrell, 1988). Different habitat conditions and feeding strategies in both aquatic and terrestrial habitats can explain the observed variability in adult body size across sampled sites (Nobili and Accordi, 1997). As pointed out by several studies, variation in life-history traits among populations of the same species often arises from differences in environmental variables influencing the genetically fixed growth pattern (Özdemir et al., 2012; Altunışık and Özdemir, 2013). Natural permanent aquatic systems and drinking troughs vary markedly in size, shape, and physical heterogeneity, providing diversified spatial and trophic resources for amphibians (Haddad and Prado, 2005; Vieira et al., 2009). Natural permanent aquatic systems are relatively deep and open habitats containing a vertical component characterized by specific aquatic micro-habitats at different depths (i.e. the shoreline, the water column, the water surface and the bottom) (Schabetsberger, 1993). Such micro-habitat heterogeneity and abundance of prey may reduce intraspecific competition and facilitate individuals in gathering adequate food supply to grow (Svanbäck and Bolnick, 2005). Contrarily, drinking troughs are small, usually shallow and physically closed habitats, devoid of a well-diversified vertical component and with a strict regime of water. Such ecological conditions may limit energy supply available for individuals that adapt to these peculiar conditions by likely reducing juvenile growth rate (Nobili and Accordi, 1997). Indeed, the absence of deep waters, overcrowding and low densities of prey may increase resource overlap, bringing individuals in direct competition (Griffiths, 1996). Females, in particular, may be more affected by trophic competition since they spend more time in water to increase their fecundity and to accumulate fat reserves for egg production (Amat et al., 2014). However, such potential high degree of intraspecific competition may not be exacerbated by other biotic processes (i.e., interspecific competition and predation) since in our study drinking troughs *L. vulgaris meridionalis* occurred without any other newt species as a potential competitor and play the role of top predator (Schabetsberger and Jersabek, 1995). Moreover, we have to consider that newts have aquatic habits during the breeding period and adopt a terrestrial life during the rest of the year consuming invertebrates comprising a large part of their diet (Joly and Giacoma, 1992; Schabetsberger and Jersabek, 1995; Vignoli et al., 2009). Different feeding conditions in terrestrial habitats in proximity of aquatic systems may further explain the observed variability in adult body size. Indeed, our study drinking troughs were often surrounded by open and dry habitats (i.e., boulders, rocky outcrops, pastures, and arable lands) whereas natural permanent aquatic systems were usually immersed in dense vegetation (i.e., hygrophilous woods, heaths, meadows and the edges of cultivated lands) with abundant leaf litter where newts can easily find abundant resources (Jehle, 2000; Schabetsberger et al., 2004).

Relationships among Italian smooth newt demography, drinking trough characteristics and management

The Italian smooth newt shows specific habitat requirements especially for the aquatic habitats (Vignoli et al., 2007a, b; Hartel et al., 2014) and our findings corroborated this suggestion. Indeed, *L. vulgaris meridionalis* populations were not uniformly distributed among the study sites but their occurrence and abundance were strongly related to some drinking trough features. A strong, positive effect of mud bottom and dense aquatic vegetation was observed on the presence of the species. Our findings are likely explained by the ecology and behavior of the species concerned. Mud bottom in drinking troughs with dense aquatic vegetation probably provide optimal conditions for life cycle, feeding and reproduction of Crustacea (Cladocera, Copepoda and Ostracoda) and aquatic insect larvae comprising a large part of the Italian smooth newt diet both at the adult (Griffiths, 1996; Vignoli et al., 2009) and larval stage (Fasola and Canova, 1992). Mud bottom makes also drinking troughs particularly suitable for hibernation that sometimes takes place into the water under the substrate (Bell, 1977; Razzetti and Bernini, 2006). We also confirmed the importance of aquatic vegetation as a recognized predictor of newt presence as suggested by Denoël and Lehman (2006). Indeed, female Italian smooth newts require dense vegetation for oviposition since they wrap eggs into leaves of aquatic vegetation in shallow water increasing their survival against predators (Razzetti and Bernini, 2006). In our study, a significant positive effect of drinking trough connectivity on the Italian smooth newt presence was found. Our findings highlighted the potential negative effect of site isolation due to habitat fragmentation on amphibian occurrence (Joly et al., 2001; Cushman, 2006). It is likely that drinking trough connectivity in the immediate surroundings influences Italian smooth newt population dynamics. Indeed, more accessible drinking troughs may favor the dispersal and migration of the Italian smooth newt from unfavorable sites and thus the persistence of the species at a local scale.

We also found that intensive management negatively affect the presence of the species. As suggested by previous studies, fill-in phenomena, trough disrepair and consequent permeability loss, tapping of wells for safety measures, the construction of architectural fences, the introduction of ornamental alien species (i.e., *Carassius auratus*), mechanical and chemical cleaning make drinking troughs unsuitable habitats for the continuous persistence of the species (Garcia-Gonzalez and Garcia-Vazquez, 2011). As suggested by Garcia-Gonzalez and Garcia-Vazquez (2011) a proper management of drinking troughs could contribute to the conservation of the species in our study region, mitigating other habitat losses (Knutson et al., 2004; Garcia-Gonzalez and Garcia-Vazquez, 2011).

In our study, a significant relationship between drinking trough size and the occurrence of paedomorphosis was found. Paedomorphosis has been frequently observed in *Lissotriton vulgaris* in different habitat conditions (Denoël et al., 2001; Moustakas and Sotiropoulos, 2015). It is likely that in larger drinking troughs with consistent water flow, the lack of severe hydric regime fluctuations and of disturbance by any kind of exploitation, along with increased food availability (Moustakas and Sotiropoulos, 2015) could have offered habitat stability to the species, thus favoring the expression of paedomorphosis. Indeed, paedomorphosis has been shown to be favored in permanent aquatic habitats at low densities (Semlitsch, 1987) and when prey are particularly abundant (Denoël and Poncin, 2001). However, paedomorphosis have been poorly studied in low-structured habitats occupied by only one species (Fasola and Canova, 1992) and thus further studies are needed in order to identify the factors responsible for the occurrence of such phenomenon.

Our study also suggested that the abundance of *L. vulgaris meridionalis* was strongly dependent on forest extent in the landscape surrounding drinking troughs. As suggested by Dodd (2010), amphibians often require a variety of vegetation type around the breeding site and the structure of vegetation is more important than the species composition. The preference showed by the species for drinking troughs located in or close to enclosed canopy is probably related to the availability of suitable terrestrial habitats for the post-breeding season (Griffiths, 1996). The species, in fact, is known to prefer sunny and well-vegetated areas characterized by moist or humid sites containing cover, surface debris, subsurface burrows, leaf litter, and dead wood where it can easily find food, shelters and hibernation sites (Jehle, 2000; Schabetsberger et al., 2004). Hibernating sites, in particular, are usually very close to reproduction sites (10-60 m) (Razzetti and Bernini, 2006). A dense forest canopy also does not affect thermal regimes, thus reducing the risk of drying, increasing prey availability and limiting disturbance and the variation of abiotic factors (e.g. temperature, humidity) (Dodd, 2010). Our findings are in agreement with deMaynadier and Hunter (1995) that documented the negative impact of clearcutting of mature forests on the abundance of amphibians due to the warmer, drier conditions detected in cutover stands. Humid terrestrial environments also facilitate newt movement compared to well-drained ones (Joly and Miaud, 1993).

Our study demonstrated that Italian smooth newts use natural permanent aquatic systems but also small artificial aquatic habitats such drinking troughs as suitable feeding, sheltering and breeding sites. Indeed, drinking troughs, in combination with natural permanent aquatic systems, seem to support spatially extent Italian smooth newt populations. Of course, population size in such artificial aquatic systems is usually much smaller than in natural permanent aquatic systems but it can be sufficient to maintain viable demes at least in the short-medium term facilitating dispersion processes, colonizations of new areas and recolonizations subsequent to potential catastrophe events (Denoël, 2004). To the best of our knowledge, there are no other demographic studies on *L. vulgaris* comparing populations from natural and artificial water systems (but see Nobili and Accordi, 1997 and Di Giuseppe, 2012 for preliminary data).

In the light of our findings, we concluded that drinking troughs, built as water reservoirs for the Maremmana cow breeding, may be of particular importance for the conservation of the Italian smooth newt at a local and regional scale in our country. This demonstrates that the presence of livestock not always have a negative effects on amphibian development and survival, as traditionally suggested (Jansen and Healey, 2003; Knutson et al., 2004). Indeed, the drinking trough structure, and in particular its elevation above the ground, avoids newts to experience the disturbances associated with grazing and direct livestock access to water observed in artificial and natural ponds. In drinking troughs, newts do not face the deterioration of water quality (i.e., high levels of nitrogen and phosphorus, and low oxygen concentrations) and of aquatic and emergent vegetation caused by livestock wading and defecating that determine an adverse environment for amphibian eggs and tadpoles (Knutson et al., 2004).

Braun (2006) suggested that artificial habitats similar to our drinking troughs (i.e. tanks filled with water) are not likely colonized by amphibians since they remain ecologically distinguishable from all natural sites because of their smaller sizes, constant hydroperiod, scarce aquatic vegetation and vertical walls. Contrarily, we consider drinking troughs as stable environments highly suitable for amphibian reproduction and representing crucial stopover points for individuals moving among wetlands. They may also represent hot spots for polyphenism that favors the adaptive intraspecific variation of the species increasing its fitness (Ryan and Semlitsch, 1998).

Conservation remarks

Although preventing the modernization of agriculture is not possible and would be undesirable from a socioeconomic viewpoint, the identification and protection of key sites related to the persistence of traditional husbandry may be crucial for amphibian survival (Garcia-Gonzalez and Garcia-Vazquez, 2011), especially in a period that is witnessing major changes in local landscapes, due to land use changes, land abandonment, and climate change (de Chazal and Rounsevell, 2009; Mantyka et al., 2012).

The relationship between traditional Maremmana cow breeding and amphibian conservation found in our study stressed the strength of the bond between economic activities and nature preservation. However, traditional cattle rearing has been declined in the last 50 years in Italy and the trend is still continuing (Bignal and McCracken, 2000; Cocca et al., 2012). In this scenario, drinking troughs, no longer needed for their original purposes, will be progressively neglected in their management losing their role as suitable habitats for amphibians. For all these reasons, there is a clear need to achieve a consensus that traditional cattle rearing practices and amphibian conservation are mutually beneficial and should be preserved (Dennis, 1998). Researches on the beneficial effects of traditional cattle rearing practices on amphibian conservation are therefore required and further experimental studies and demonstrations are urgently needed for ecological as well as for social reasons (Dennis, 1998).

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Figure 1. Location of artificial and natural permanent aquatic systems (N=58) sampled in the study site (Tolfa Mountains, Latium region, Central Italy). Symbols: light blue circles = natural permanent aquatic systems; red squares = drinking troughs.

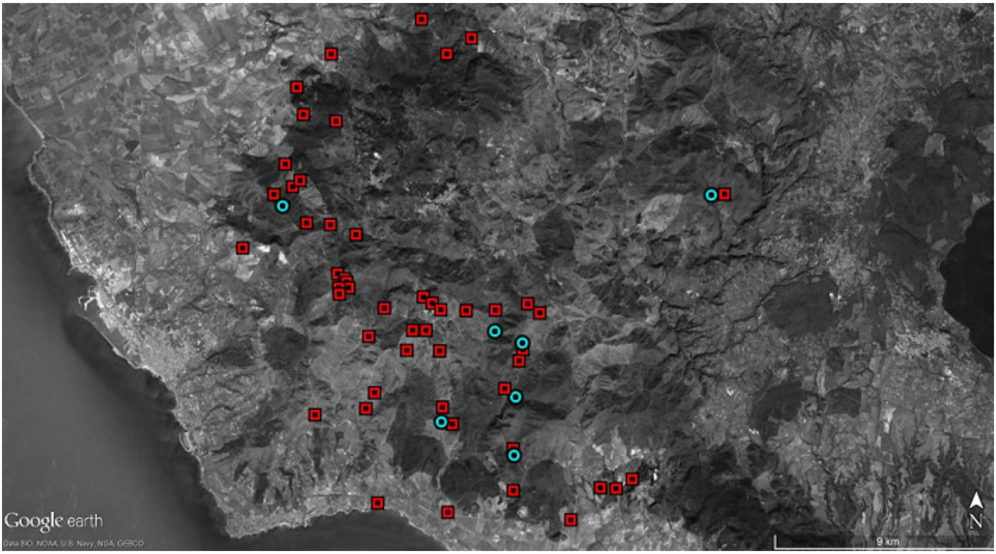

Figure 2. Adult body size (mean ± SD) at the study drinking troughs and natural permanent aquatic systems.

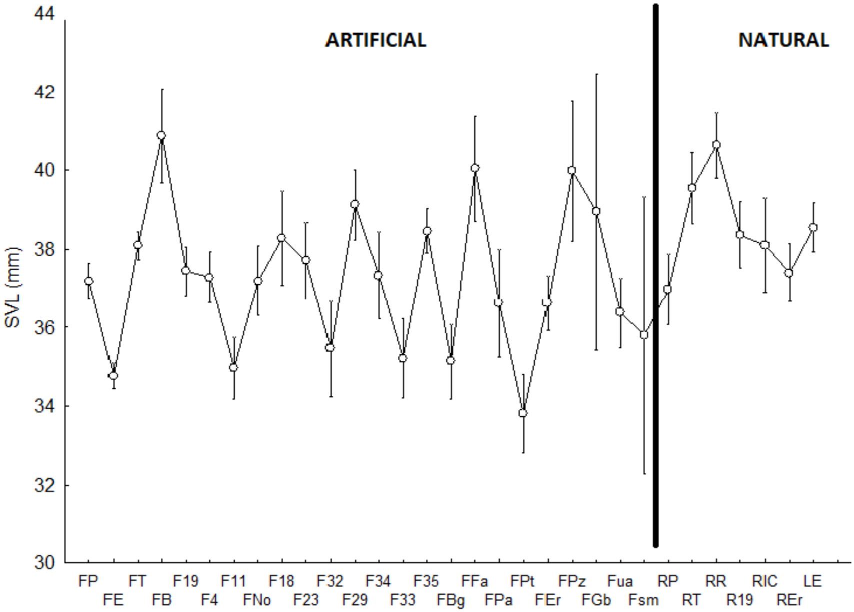

Figure 3. Interaction effect of site typology and sex (SITE TYPE*SEX) on newt body size (SVL). Newts showed clear body size dimorphism (i.e. females larger than males) and were larger in natural aquatic habitats in respect to artificial habitats.

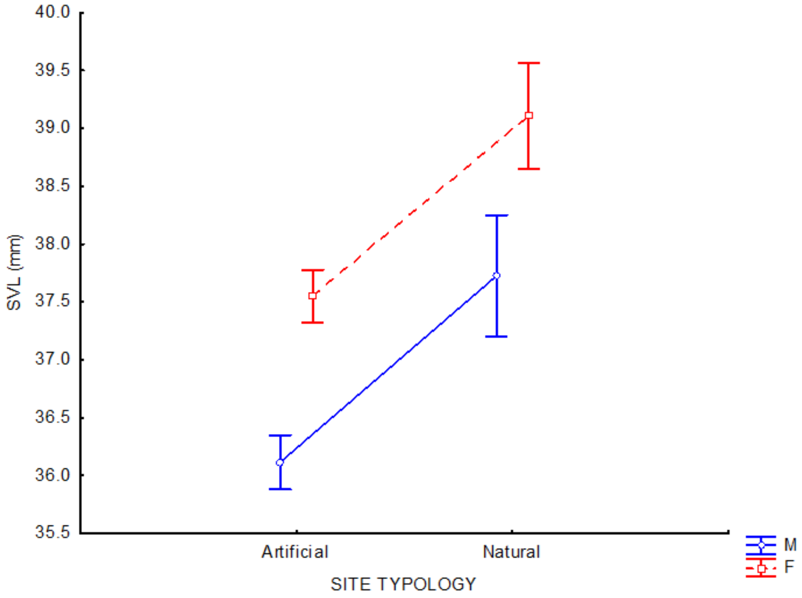

Table 1. For all the study drinking troughs we reported data on occurrence (P: presence/absence), abundance (PE: population estimate – n individuals), and density (D: ind/m3) of *L. vulgaris meridionalis* populations, and drinking troughs characteristics as volume (V; m3), type of vegetation covering (Vf: floating vegetation; Vs: submerged vegetation; Ve: emerged vegetation), intensity of management activities (M; 0=no management; 1= moderate management with vegetation control and surface cleaning once a year; 2= intensive management resulting in periodic cleaning and disinfection with chemicals), and landscape features (FC: percentage of forest cover). Asterisks on drinking trough ID indicate the occurrence of paedomorph individuals.

ID	P	PE	V	D	V	M	FC
F01	0	0	4.33		Vf-Vs-Ve	0	60
F02	0	0	5.07		Ve-Vs	0	5
F04*	1	66	8.29	7.96	Vf-Vs	1	20
F11*	1	77	7.71	9.99	Vf-Vs	1	15
F12	0	0	1.52		Vf-Vs-Ve	0	35
F13	0	0	4.78		Vs	1	15
F14	0	0	8.31		Vf	2	20
F16	0	0	5.67		None	2	20
F17	0	0	6.28		Vs	2	7
F18	1	20	9.94	2.01	Vf-Vs	1	30
F19*	1	72	8.1	8.89	Vf-Vs-Ve	1	65
F23	1	29	5.81	4.99	Vf-Vs	1	45
F24	0	0	14.39		None	0	40
F25	0	0	6.19		Vf	2	10
F27	0	0	16.65		Vf	1	50
F28	1	38	11.29	3.37	Vf-Vs-Ve	1	5
F29	1	37	10.83	3.42	Vs	2	65
F30	0	0	23.80		Vf-Vs	1	30
F31	0	0	10.33		None	2	35
F32*	1	56	12	4.67	Vf-Vs	1	20
F33	1	29	10.99	2.64	Vf-Vs	1	25
F34	1	24	11.97	2.01	Vf-Vs	1	40
F35	1	89	6.79	13.11	Vf-Vs	1	50
F36	0	0	1.97		Vf-Vs	1	40

F44	0	0	2.95		Vf	1	40
F45	0	0	4.34		Vf	1	50
FB	1	18	5.03	3.58	Vs	1	45
FBg*	1	34	18.72	1.82	Vf-Vs-Ve	1	30
FBo	1	17	7.85	2.17	Vs	1	10
FCh	0	0	6.79		Vs	0	30
FE*	1	132	14.21	9.29	Vf-Vs-Ve	1	65
FEr*	1	62	6.62	9.37	Vf-Vs	1	35
FFa*	1	23	53.9	0.43	Vf-Vs-Ve	1	45
FFm	0	0	5.52		Vf-Vs	1	20
FFo	0	0	24.90		Vs	2	20
FGb	1	2	2.96	0.68	Vs	1	21
FGi	1	14	1.3	10.77	Vf-Vs-Ve	1	25
FId	0	0	1.89		Vf-Vs	2	35
FMr	0	0	2.26		None	2	20
FNo	1	21	21.2	0.99	Vf-Vs-Ve	1	40
FP	1	73	4.03	18.11	Vf-Vs-Ve	1	60
FPa	1	14	4.11	3.41	Vf	1	35
FPt*	1	125	26.24	4.76	Vf-Vs-Ve	1	60
FPz	1	10	2.15	4.65	Vs	1	18
FRi	0	0	9.05		None	0	5
FSc	0	0	2.24		None	2	15
FSm	1	2	9.27	0.22	Vf-Vs	1	5
FSs	0	0	6.69		None	2	5
FT	1	214	3.88	55.16	Vs	1	70
FUa*	1	41	18.9	2.17	Vf-Vs-Ve	1	45
FVe	0	0	4.07		None	2	15

Table 2. Eigenvalues and Factor loadings from the Factor Analysis performed on all the drinking troughs filled with water. Marked loadings are > 0.700.

Value	Eigenvalue	% Tot-variance	Cumulative - Eigenvalue	Cumulative - %
1	3.277	29.791	3.277	29.791
2	1.870	17.003	5.147	46.794
3	1.558	14.162	6.705	60.955
4	1.353	12.303	8.058	73.259

Variables	Factor - 1	Factor - 2	Factor - 3	Factor - 4
Surface	0.030	**0.983**	0.021	0.086
Volume	0.066	**0.980**	0.060	0.0248
Bottom	**0.787**	-0.087	0.010	0.418
Floating vegetation	0.135	0.233	0.365	0.595
Emerged vegetation	-0.001	0.197	0.095	**0.772**
Submerged vegetation	**0.747**	0.074	0.054	0.314
Management	-0.288	0.046	0.075	**-0.748**
Connectivity	**0.752**	0.169	0.202	-0.318
Forest cover	0.147	0.030	**0.907**	0.119
Distance to forest	-0.015	-0.059	**-0.861**	0.050

Human disturbance	-0.619	-0.193	-0.170	-0.026
Expl. Var	2.255	2.105	1.780	1.909
Prp. Tot.	0.205	0.191	0.163	0.173

Table 3. Generalized linear model performed on newt occurrence. The Wald statistics and the estimates for the effects of the four factors extractred by PCA are shown. Significant effects are marked in bold.

Effect	Estimate	Standard - Error	Wald - Stat.	p
Intercept	0.159	0.348	0.208	0.649
Factor - 1	**1.242**	**0.406**	**9.355**	**0.002**
Factor - 2	0.643	0.374	2.962	0.085
Factor - 3	0.54627	0.347	2.479	0.115
Factor - 4	**0.659**	**0.343**	**3.695**	**0.055**
Scale	1	0		

CHAPTER 2

An improved microsatellite panel to assess genetic variability in the Italian smooth newt *(Lissotriton vulgaris meridionalis)*

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Summary

Habitat loss and fragmentation is threatening amphibians by increasing population isolation. However, artificial waterbodies created for livestock may contrast this phenomenon by providing surrogate habitats for amphibians. Here, we performed a genetic study on an amphibian species, *Lissotriton vulgaris meridionalis*, in a rural area where natural wetlands are disappearing and drinking troughs for cattle watering are widespread. Specifically, we tested a panel of microsatellite markers in order to identify a suitable tool for addressing conservation genetic issues of this species that is undergoing severe local declines. Twelve out of 20 tested loci produced reliable amplifications and were polymorphic. Three distinct units with a low level of gene flow were distinguished and the population genetic structuring overlapped with geographic distribution. Such loci will be useful to assess the genetic diversity of the species across multi-scale levels for its management and conservation.

Introduction

Amphibians are undergoing major global declines in the last decades because of pollution, pathogens, exotic species, UV irradiation, habitat destruction, and climatic change (Alford and Richards 1999). Habitat loss and fragmentation, consequent to agricultural intensification, have posed serious threats to many species by increasing population isolation (Beebee 2005). Indeed, intrinsic amphibian features such as meta-population structure (Alford and Richards 1999), low dispersal abilities, and strong site fidelity (Squire and Newman 2002) make them particularly vulnerable by limiting inter-population exchanges and structuring populations in distinct genetic units despite geographic proximity (Jehle and Arntzen 2002). The study of amphibian population dynamics and connectivity and the identification of demes vulnerable to genetic threats are therefore a priority from a conservation perspective, especially at a small geographic scale (Semlitsch 2000; Jehle and Arntzen 2002). In this context, microsatellite markers represent suitable genetic tools to identify populations with reduced genetic diversity, infer population structure, estimate effective population size, determine levels of migration and gene flow among populations, and investigate the effects that barriers and other landscape features have on populations (Jehle and Arntzen 2002; Beebee 2005).

The smooth newt *Lissotriton vulgaris* (Linnaeus 1758) is abundant and widely distributed in Europe (Razzetti and Bernini 2006) and is an ideal candidate for investigating regional population connectivity because it (i) has limited dispersal capabilities, (ii) exhibits high breeding site fidelity, (iii) is a highly deme-structured species (Griffiths 1996; Roth and Jehle 2016). The smooth newt is not protected by Habitat Directive although local populations seem to be undergoing severe declines, especially in Italy (subspecies *meridionalis*), following habitat destruction and fragmentation (Razzetti and Bernini 2006).

The aim of the present study was to test a panel of 20 microsatellite markers (previously developed and tested for cross-species amplification in other *Lissotriton* species and subspecies) on the Italian smooth newt, *Lissotriton vulgaris meridionalis* (Boulenger 1882), in order to identify a suitable tool for addressing conservation genetic issues of this potentially threatened species. Specifically, we aimed to evaluate the usefulness of this panel in assessing population structure and genetic variability at a local spatial scale.

Materials and Methods

Fieldwork was carried out during 2015 in a Special Protection Area (SPA, IT6030005) in Latium region (Central Italy) where natural wetlands are disappearing being no longer available for amphibian reproduction. However, drinking troughs constructed for free-ranging cattle watering are particularly widespread in the study area and may indirectly provide surrogate breeding habitats for amphibians. We collected tissue samples by tail clipping from 57 adult Italian smooth newts from three demes inhabiting drinking troughs 10 to 15 km far from each other (FB = Bandita, FP = Pianoro, FT = TorCimina. Distances (km): FP-FT = 11.7; FB-FP = 10.1; FT-FB = 15.7; Fig 1). Tail-clips were stored in 95% ethanol until analysis.

Twenty published microsatellite loci were selected and tested (Table 1). The forward primers were labelled with a fluorescent dye (Applied Biosystems). Newt tail-tips were digested using a proteinase K solution (56°C, overnight). Total genomic DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN). Simplex PCRs were performed to test each of the 20 primer pairs. Among these, 12 produced reliable amplification with polymorphic results, whereas the remaining primers were discarded because of no amplification, low signal, or many aspecific signals (Table 1). In order to optimize time and cost of our analyses, we ran three multiplex PCRs (M1, M2, M3; Table 1) using Qiagen Multiplex PCR Kit in 8 µL mix solution composed by 3.50 µL HotStarTaq Master Mix, 0.70 µL Qsol, 1 µL DNA, 0.20 µL each primer (10 µM), brought to volume with H_2O. Amplification conditions for all loci were carried out setting an initial denaturing at 95 °C for 15 min, 40 cycles of denaturing at 94 °C for 30 sec, annealing temperature of 55 °C for 1.30 min, and 72 °C extension for 1 min, and a final 10 min extension at 72 °C. Two replicates for each sample at each locus were performed. PCR products were electrophoresed with internal size standard (GeneScan 1200 LIZ, Life Technologies) in an ABI

3130XL sequencer and allele sizes were scored using the software GeneMapper v.4.0 (Life Technologies).

We checked newt genotypes for the presence of null alleles (MICRO-CHECKER, Van Oosterhout *et al.* 2004), Hardy-Weinberg equilibrium within populations (GENEPOP v.4.2, Rousset 2008), estimated average number of alleles per locus, allele frequencies, expected and observed heterozygosities, and genetic distances between the samples, clustered through Principal Coordinates Analysis (PCoA) (GENALEX v.6.5, Peakall and Smouse 2006, 2012). We then inferred population genetic structure and distinguished the clusters of populations by means of Bayesian procedures (STRUCTURE v.2.3.4, Pritchard *et al.* 2000). For evaluation of optimum number of populations (K), a simulation was coordinated using parameters K (1-3) with a random start for each K value and 5 independent runs (200,000 iterations following a burn-in period of 20,000) and assuming ΔK value, which takes into account the shape of the log likelihood curve (Evanno *et al.* 2005).

Results and Discussion

Twelve out of 20 tested microsatellite loci produced reliable amplifications. All loci were polymorphic except LVG-388 in FB and FP populations, and LVG-210 and Lm_013 in FT population. A similar low-level of successful cross-species amplification has been documented in other *Lissotriton* species (Johanet *et al.* 2009; Nadachowska *et al.* 2010). This was not surprising since close related amphibian taxa (i.e. congeneric species and subspecies) often showed a cross-species amplification success rate lower than expected (Primmer and Merilä 2002). This is probably due to the intrinsic characteristics of urodelan genome (i.e., large-sized and complex) resulting in an amplification success that decreases significantly with its increasing size (Garner 2002). Furthermore, microsatellites are usually found in noncoding regions with high substitution rates positively correlated with genetic divergence time (Primmer and Merilä 2002). Thus, the detected low cross-amplification success may be also explained by the fact that *Triturus* genus is no longer considered monophyletic and *Triturus* species diverged long time ago (i.e. more than 60 Mya; Steinfartz *et al.* 2007).

The number of alleles per locus ranged from two (LVG-388) to 18 (Lm_488 and Lm_749) and the expected heterozygosity from 0.046 (LVG-388) to 0.810 (Lm_521) (Table 1). After Bonferroni correction, the observed and expected heterozygosities did not differ significantly among the loci. Overall, the average number of alleles ranged from 4.333 (FP) to 5.667 (FB) and the expected heterozygosity from 0.465 (FT) to 0.600 (FB) (Table 2). The estimated deviations from Hardy-Weinberg equilibrium were not significant for all loci. The three populations exhibited good levels of genetic variability as for total values of average allele number (4.806), expected heterozygosity (0.538), and percentage of polymorphic loci (88.89 %) (Table 2). The detected genetic diversity suggests that the study populations may face a low risk of extinction by readily adapting to changing environmental conditions (Reed and Frankham 2003).

Both PCoA and Bayesian analyses distinguished three distinct units, differentiated and isolated from each other with a low level of gene flow (Fig 2A, B). Such population genetic structuring overlapped with geographic distribution with a highest differentiation among the farther demes. As established from K distribution, the graphical method detected the highest value of ΔK at $K = 3$ indicating optimum number of clusters (Fig 2B), with a first split between FB-FP and FT ($K = 2$, Fig 2B). Clustering of the populations was performed on the basis of genetic similarity among the groups irrespective of geographical locations of sampling. Differentiation between FB and FP ($F_{ST} = 0.081$) was low compared to what has been estimated for FT. Indeed, FT was more geographically and genetically isolated from the others showing the highest pairwise F_{ST} values (0.129 with FB and 0.132 with FP). Amphibian populations often exhibit a high degree of spatial structure, particularly when inter-population distances exceed several kilometres (Shaffer *et al.* 2000). In this case, the population differentiation positively correlated with the distance among breeding sites. However, the observed pattern could be likely influenced also by landscape features (i.e. barriers and corridors) that facilitate/impede newt dispersal.

The set of markers, developed for other *Lissotriton* species-subspecies and optimized in the present study, represented a reliable tool for population genetic analyses in the subspecies *L. v.*

meridionalis. Such loci had a good resolution even at a fairly fine-scale and will help to asses Italian smooth newt genetic diversity and understand its genetic structure across multi-scale levels, from meta-populations to full geographical range. These markers will be helpful in planning strategies for effective management and conservation of the species.

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Tab 1. Twenty published microsatellite loci, selected and tested on collected newt samples.

Locus	Fluorescent dye	Sequence (5'-3')		Locus size range	Na	Ho	He	Multiplex
Lm_013*	HEX	F	CTTGGTTCCCAGTGAGGAGA	156-164	3	0.201	0.259	M3
		R	GCAAGCCATCCCAAAGTAAG					
Lm_488*	PET	F	CAGGCAGGGTATTTGCGTAG	236-522	18	0.864	0.783	M2
		R	GGTCATTTCCACAACAAGCTC					
Lm_521*	FAM	F	CATACGGGCACTGAGGTGAT	236-320	13	0.797	0.810	M1
		R	GCACAGACATTGATGGCAAA					
Lm_528*	NED	F	CTGGCTTGAAATGCCTTCAT	Many aspecific signals				
		R	AGGGCAGGGCTATACGTCTT					
Lm_632*	FAM	F	CAGAGCAATTTCTAGGCAAGG	176-244	14	0.780	0.680	M2
		R	GGCGCTATATCAAACTGCAA					
Lm_749*	PET	F	CCATGGTGGTAGAATAAATGGAA	214-542	18	0.802	0.738	M3
		R	AAGACCATTCTTTCTGAGGTATCC					
LVG-210/EU568357#	HEX	F	ATGAGCCAACACCATGTCTG	222-228	4	0.190	0.156	M1
		R	TGATGTCCTTGGGATCCTTG					
LVG-250/EU568353#	HEX	F	GCTTGGGAGAGCCCTATCTT	Many aspecific signals				
		R	CCAATAGTTTAATCTCTGGCAAATG					
LVG-267/EU568359#	NED	F	CTTGTCTTTAAGGGCCCAAGT	Many aspecific signals				
		R	TCTGCACAACAACACAAGACC					
LVG-303/EU568355#	FAM	F	AGTAGTGATCAGCGGCACAGT	Low signal				
		R	CGCTAGGAGGCAAGATCCTAT					
LVG-388/EU568352#	PET	F	GTGGGTGGTAGGCCAGAATAC	157-159	2	0.050	0.046	M1
		R	CCCATGAACAACCCATTAGG					
LVG-398/EU568356#	NED	F	ACCAGATATGTTCCGCTTCCT	126-144	6	0.649	0.649	M3
		R	TTGCAAATAAAATAACGCTACCC					
LVG-449/EU568358#	HEX	F	AGGTCAGATGGGTTTCCTTGT	Low signal				
		R	GATCCGTAGTCAGACGCGTTA					

LVG-542/EU568354#	HEX	F	ACAACACAGCCAGATTTCCAA	139-151	5	0.537	0.481	M1
		R	TACAATGATCCTTTCGCCTTG					
Th09+	PET	F	CGACACTCGCAAATCAATC	137-177	10	0.640	0.735	M2
		R	ATTATTATTTCCACACACGTAAACAT					
Th14+	HEX	F	CCCACTGGGAATCTGAGAAA	No amplification				
		R	TGCCATATTGTTGAGGACCA					
ThCa14+	HEX	F	ACCTGCTGACAGTGCAAATG	No amplification				
		R	CGATTGATCATGGGACACGTA					
Tv12+	FAM	F	AACCGGGTACACCTACATCA	Many aspecific signals				
		R	CTCACCTTTGTAATGGCCTCT					
Tv3Ca9+	NED	F	AAATAACTTGTGATTGGGTCATTT	79-111	3	0.532	0.504	M2
		R	TGCATATATACTGTATGTTTACTGCAA					
Tv3Ca19+	HEX	F	CCTCCACGAGGTTACTGCAC	62-100	6	0.391	0.616	M3
		R	GACCATTTCAGACACTCATTACG					

For each locus is shown: fluorescent dye used, microsatellite sequence, size range, allele number (Na), observed (Ho) and expected (He) heterozygosity, multiplex PCRs performed.

*: *Lissotriton montandoni*, Nadachowska et al. 2010

#: *Lissotriton vulgaris graecus*, Sotiropoulos et al. 2009

+: *Lissotriton helveticus* and *Lissotriton vulgaris*, Johanet et al. 2009

Tab 2. Estimates of population genetic diversity at 12 microsatellites.

Pop	**N**	**%P**	**Na**	**Pa**	**He**	**Ho**	**HWE**
FB	17	91.67%	5.667 (0.899)	2.000 (0.685)	0.600 (0.073)	0.581 (0.084)	0.002
FP	20	91.67%	4.333 (0.595)	1.333 (0.355)	0.549 (0.076)	0.556 (0.084)	0.154
FT	20	83,33%	4.417 (0.763)	1.167 (0.458)	0.465 (0.089)	0.472 (0.102)	0.192
TOT	57	88,89%	4.806 (0.440)	-	0.538 (0.046)	0.536 (0.051)	0.003

Sample size (N), percentage of polymorphic loci (%P), allele number (Na), private alleles (Pa), expected heterozygosity (He), observed heterozygosity (Ho) and Hardy-Weinberg equilibrium (HWE); in brackets, standard error values.

Figure legends

Fig 1. Satellite map of the study area with localities for the three sampled *Lissotriton vulgaris meridionalis* populations inhabiting drinking troughs (FB = Bandita, FP = Pianoro, FT = TorCimina).

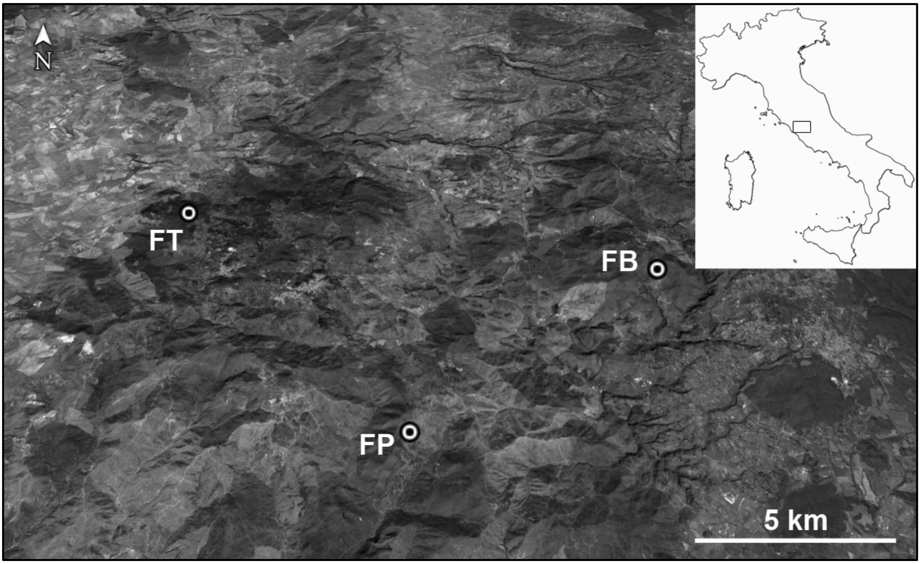
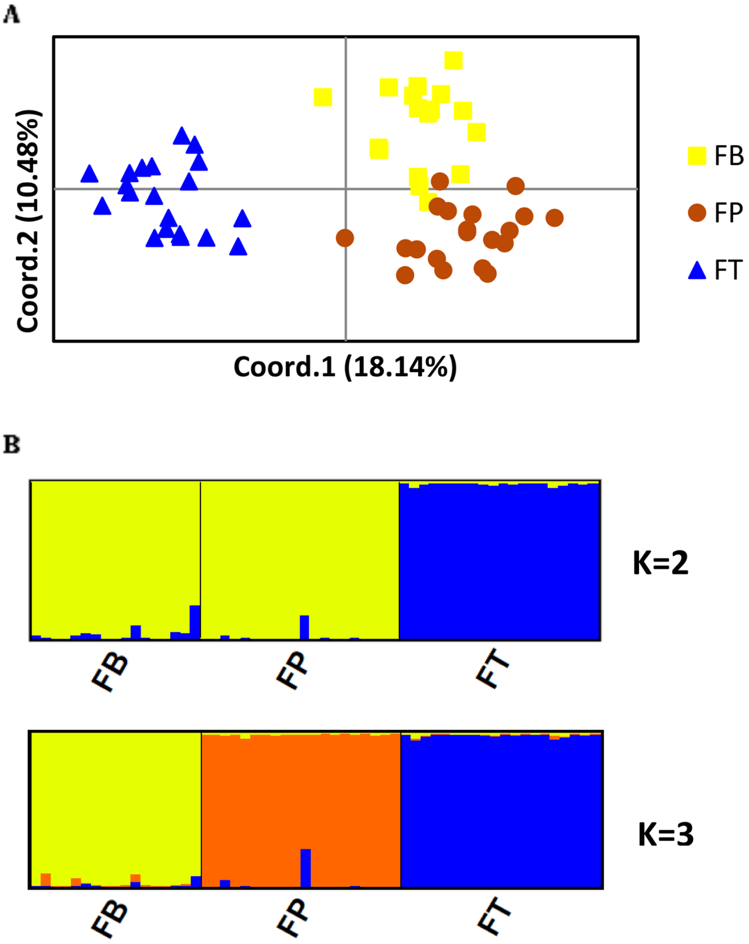


Fig 2. A. Principal Coordinates Analysis (PCoA) of 57 smooth newt samples. B. Bayesian clustering results regarding three smooth newt populations inhabiting drinking troughs (FB = Bandita, FP = Pianoro, FT = TorCimina); (K = 1-3, pop-info = 0).



CHAPTER 3

Fine-scale spatial genetic structure and dispersal among Italian smooth newt *(Lissotriton vulgaris meridionalis)* breeding populations in a rural landscape

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Introduction

Nowadays, anthropogenic land use change is by far the most severe driver of habitat loss and fragmentation, leading to species extinctions and reduction of worldwide biodiversity (Lindenmayer and Fischer 2013). There are few landscapes on Earth that have not been altered by humankind's interaction with nature (Bignal and McCracken 2000). In this regard, Crutzen (2002) coined the term "anthropocene", a new geological era during which biodiversity patterns cannot be explained without considering human activities. This is particularly evident in the Mediterranean basin, a biodiversity hotspot, in which the current mosaic of agricultural lands, traditional pastures, evergreen woodlands and maquis habitats, is the result of anthropogenic disturbances over millenia (Blondel, 2006; Sokos et al. 2013). Indeed, in the developing world, agricultural systems have supported high levels of biodiversity likely due to their retention of spatial heterogeneity (Curado et al. 2011). Specifically, in extensive farmlands, the traditional and low intensity land management allowed the establishment of species-rich assemblages (Stoate et al. 2009).

In the second half of the 20th century, the agricultural expansion and intensification determined habitat loss and fragmentation, spatial homogenization and, ultimately, a reduction in biodiversity also in rural systems (Tilman et al. 2002). Among animals, amphibians are demonstrated particularly threatened by this phenomenon (Beebee and Griffiths 2005) because of their peculiar characteristics, as relatively low vagility (Bowne and Bowers 2004) and strong site fidelity (Kusano et al. 1999), that amplifies the effects of habitat fragmentation, habitat loss and degradation, and edge effects (Houlahan and Findlay 2003; Cushman 2006). Agricultural expansion and intensification are increasing the disappearance of natural habitats suitable for amphibian foraging, overwintering and breeding (Garcia-Gonzalez and Garcia-Vazquez 2011). However, artificial facilities, associated with traditional agriculture (irrigation) and husbandry (cattle watering), and originally connected to natural water systems, may represent important surrogate breeding habitats for amphibians (Knutson et al. 2004; Garcia-Gonzalez and Garcia-Vazquez 2011). Indeed, the ability of amphibians to colonize artificial aquatic sites (e.g. tanks, drinking troughs and reservoirs) has been well documented worldwide (Petranka et al. 2003; Rannap et al. 2009; Lannoo 2014).

Isolated populations may be subject to local extinction (Frankham 2005) whose genetic and demographic rescue may become impossible. Currently, not much is known on the dispersal patterns and the geographical scale at which gene flow is limited in amphibians. Studies clarifying the dynamics of populations are therefore needed, in addition to demographic researches, in order to provide appropriate data for management and conservation decisions in response to increasing anthropogenic habitat modification (Manel et al. 2003; Purrenhage et al. 2009). In this context, properly managed drinking troughs may represent the only permanent water points available as breeding sites of amphibians playing an important role in sustaining viable populations of threatened species (Garcia-Gonzalez and Garcia-Vazquez 2011). Genetic data have become increasingly important for conserving and managing threatened and endangered species (Allendorf and Luikart 2007). Microsatellite markers have been employed in studies of population structure to estimate the effects of anthropogenic disturbance, being sensitive indicators of change in genetic variability and dispersal rates (Scribner et al. 1994). Indeed, identifying populations with reduced genetic diversity is critical for determining extinction risk (Quattro and Vrijenhoek 1989; Saccheri et al. 1998), whereas differences in dispersal among populations may result in genetic patterns serving as signals of disruptions in regional population connectivity (Purrenhage et al. 2009).

The Italian smooth newt *Lissotriton vulgaris meridionalis* is an example of a species potentially threatened by the destruction and fragmentation of terrestrial and aquatic habitats by rural and urban development (Razzetti and Bernini 2006). It is a small-bodied member of the Salamandridae family, one of the seven subspecies included in the *Lissotriton vulgaris* taxon (Babik et al. 2005). The species is widely distributed south of the Alps, in southern Switzerland, northern and central Italy, western Slovenia and Istria (Razzetti and Bernini 2006), at elevation from sea level up to 1700 m (Appennines) (Razzetti and Bernini 2006). The Italian smooth newt usually lives in hygrophilous woods, heaths, meadows and the edges of cultivated lands, and needs water bodies only for reproduction (Razzetti and Bernini 2006). Reproduction takes place during spring months (Vignoli et al. 2007) in ditches, pools, drinking troughs and cisterns, located in both open and forested areas. It has also been recorded in human-modified environments, although its presence is sometimes limited by agriculture activity (Ildos and Ancona 1994). The species is not protected by Directives 92/43/EEC and 97/62/EU, although populations are locally declining, especially in Italy (Razzetti and Bernini 2006). The vitality of Italian populations seems to be be closely linked to the conservation of small and temporary wetlands that allow egg-laying and larval development (Nobili and Accordi 1997; Pizzuti Piccoli 2008).

Here, we addressed the role of drinking troughs in maintaining spatial connectivity and genetic variability at a fine geographic scale among Italian smooth newt populations in a rural area (Latium region, central Italy) characterized by traditional husbandry and agriculture. Specifically, information drawn from microsatellite genotypes were used to (i) examine the amount of genetic diversity whithin and among artificial and natural breeding sites; (ii) infer population structure in the study area; (iii) evaluate the level of gene flow among demes; iv) establish whether drinking troughs may provide alternative and/or complementary aquatic habitats for amphibian conservation in rural landscapes.

Materials and Methods

Population sampling

Fieldwork was carried out in the Comprensorio-Tolfetano-Cerite-Manziate (Province of Rome, Latium Region, Central Italy) a Special Protection Area (SPA, IT6030005), spanning 70,000 ha, included in the European Ecological Natura 2000 network. In this area, natural wetlands are scarce, being dried and drained for agricultural purposes, and characterized by a strong seasonality with short hydroperiod, especially due elevated temperatures reached in the summer months (June, July, August) and irregular rainfalls. The study site shows a rural profile interested by traditional husbandry where troughs for livestock watering (cattle, horses and donkeys) are particularly common and widespread. The presence of free-ranging cattle maintains open habitats that otherwise would be replaced by wooded areas and provides additional aquatic resources represented by small artificial water bodies built by farmers to facilitate their management. Drinking troughs, originally connected to natural brooks or ponds but nowdays usually isolated from natural water bodies due to the disappearance of these latter, are widespread and located in a landscape characterized by a mosaic of pastures, crops and woods. All the potential breeding aquatic systems (natural and artificial) were identified in the study area by performing interviews to local landowners and by locating them from orthophoto by using Google Earth Pro. Exact localities were determined using a global positioning system in the field or large-scale maps of the area. Overall, we recorded 7 natural aquatic habitats and 70 drinking troughs over a 18,000 Ha area (0.004 trough/Ha). Finally, we identified directly in the field 51 active drinking troughs and 7 natural water bodies. We selected 14 troughs based on newt presence and distribution over the study area in order to achieve a good geographic representation of the area and minimize spatial autocorrelation. Moreover, we individuated five artificial-natural water body pairs, each consisting of a drinking trough built in the immediate surrounding (<500m) of a natural brook/pond. These pairs represent a good model to study the genetic diversity of newt populations inhabiting artificial vs. natural sites that are connected to each other and most likely originated form the colonization of artificial water body from the paired natural site. All the seven natural aquatic systems (1 pond and 6 brooks) and the selected 14 troughs were sampled during the breeding season (March-May) in 2015-2016 years. We surveyed drinking troughs by dip netting (Arntzen 2002), whereas in the natural sites newts were visually located and captured by hand or nets. We collected tissue samples by tail-clipping from at least 20 adult individuals from each aquatic site. Samples were stored in ethanol 95% at room temperature until genetic analysis. Newts were then marked by a photograph of the ventral pattern and released after the sampling. Since cohorts can vary in genetic composition, we avoided sampling the same aquatic system at different times during the year (Scribner et al. 1993).

Laboratory analyses

DNA extraction and microsatellite amplification

We genotyped 464 individuals. In this study we employed 12 microsatellite loci tested and optimized previously for this species (Buono et al. 2017, in press). To optimize the costs and time spent on the analyses, we ran three multiplex PCRs (M1 included Lm_521, LVG-210/EU568357, LVG-388/EU568352 and LVG-542/EU568354; M2 included Lm_488, Lm_632, Th09 and Tv3Ca9; M3 included Lm_013, Lm_749, LVG-398/EU568356 and Tv3Ca19). Tail clips were digested overnight at 56 °C in 180 µl lysis buffer with 20 µl proteinase K. Total genomic DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN) and QIAcube robot. DNA extracts were used as templates for amplification of microsatellite loci. PCRs were performed using Qiagen Multiplex PCR Kit in an 8 µL mix composed of 3.50 µL HotStarTaq Master Mix, 0.70 µL Qsol, 1.50 µL DNA, 0.20 µL each primer (10 µM), brought to volume with H_2O. Amplification conditions for all loci included an initial denaturing at 95 °C for 15 min; 40 cycles of denaturing at 94 °C for 30 sec, annealing temperature of 55 °C for 1.30 min, and 72 °C extension for 1 min; and a final 10 min extension at 72 °C. Two replicates for each sample at each locus were executed.

Forward primers for each locus were 5′-labelled with a fluorescent dye for detection (Applied Biosystems). PCR products were electrophoresed with internal size standard (GeneScan 1200 LIZ, Applied Biosystems) in an ABI 3130XL sequencer and allele sizes were scored using the software GeneMapper v.4.0 (Applied Biosystems). One marker (Lm_013) was finally excluded from the data analyses because of a large amount of missing data occured in almost all populations, likely due to high mutation rates at primer binding sites.

Within-population patterns of genetic diversity

Genetic diversity indices (number of alleles, effective number of alleles and number of private alleles) were calculated for each population by GenAlex v.6.5 (Peakall and Smouse 2012). Allelic richness was then calculated using Fstat 2.9.3 (Goudet 1995). This is a better estimate of genetic diversity than allele number because it takes into account uneven sample sizes. Fstat used a random sample of 16 individuals from each population. Arlequin v.3.5.2 (Excoffier et al. 2005) was used to estimate observed (H_O) and expected (H_E) heterozygosities and to test genotypic frequencies at each population and locus for statistically significant deviations from Hardy–Weinberg equilibrium (HWE). We used a Monte Carlo approximation of the Fisher's exact test (Guo and Thompson 1992) and a standard Bonferroni correction for multiple comparisons (adjusted P value < 0.0001, for a table-wide significance of $\alpha = 0.05$). The Markov chain algorithm was run for 100,000 steps following 10,000 dememorization steps. We also estimated the pairwise probability of linkage disequilibrium using a Fisher's exact test implemented in Genepop v.4.2 (Rousset, 2008) with 10,000 steps following a 1000 step dememorization.

Among-population patterns of genetic diversity

Fstat 2.9.3 (Goudet 1995) was used to perform a global test of overall population differentiation (not assuming HWE within populations). This test permutes genotypes among populations to create a null distribution for comparisons with observed levels of population differentiation (Goudet et al. 1996). Degree of population subdivision from multilocus estimates of F_{ST} (Weir & Cockerham 1984) for all population pairs was determined. Pairwise significance tests for F_{ST} (Goudet et al. 1996) were performed by permutation and resampling of multilocus genotypes among pairs of samples. Performing 10,100 randomizations allowed for a table-wide significance at the 5% nominal level after standard Bonferroni corrections (adjusted P value = 0.0001). We compared the average F_{ST} values across sites by means of one-way ANOVA in order to reveal differences between populations. Moreover, we analyzed the F_{ST} values of demes representing site pairs (one natural and one artificial site) by means of General Linear Models to evaluate genetic differentiation between geographically closely related demes inhabiting different habitats. We used F_{ST} values as dependent variable, site typology (TYPE: natural vs. artificial, categorical predictor), site pair membership (PAIR, categorical predictor), as well as the interaction term TYPE*PAIR as independent variables.

Moreover, in order to understand if artificial breeding sites support independent and distinct populations or, conversely, if they act as stopovers and are linked by dispersal events, we determined the degree of gene flow among demes in terms of success of immigrants by using *N*m estimates, derived from the inbreeding coefficient F_{ST} (Wright 1943). *N*m was developed by Dobzhansky and Wright (1941) as a measure of genetic introgression, not as an estimate of net migration. Critiques of *N*m as a migration measure are justified (Purrenhage et al 2009). Indeed, *N* represents effective breeding size while m measures the extent of population replacement in each generation. Thus, *N*m is the measure of the genetic turnover of demes, and it is scaled to allow easy comparison across populations.

Pairwise population linearized F_{ST} values (Slatkin 1995) were correlated with geographical distances to test for patterns of spatial subdivision and isolation by distance (IBD) using a Mantel test with 1000 randomizations (implemented in GenAlex v.6.5). We perfomed two tests for IBD separately, on i) all populations, and on ii) populations with exclusion of potential outliers. Populations showing higher genetic distance despite similar geographic distance in respect to the others were defined as outliers. We then used the population F_{ST} estimates in a Principal Coordinates Analysis (PCoA), to examine genetic clustering of populations throughout the study area.

Bayesian estimates of genetic structure

Bayesian clustering approaches simultaneously aim to identify the number of clusters and to assign probabilistically either individuals (versions without admixture models) or a fraction of their genome (with admixture models) to identified clusters such that Hardy-Weinberg and linkage disequilibria are minimized. Numerous models and software programs have been developed to achieve these objectives. Although their goals are similar (i.e., describing the genetic structure in each subpopulation using a joint probability distribution over the observed loci), the explicit model assumptions, fine details, and computational strategies vary among approaches and may lead to differences in performance.

We performed Bayesian clustering analysis to characterize the population genetic structure of selected aquatic systems across the study area and assess the geographical scale of population differentiation. At first, we used the software STRUCTURE v2.3.4 (Pritchard et al. 2000) that identifies clusters of genetically similar individuals from multilocus genotypes without prior knowledge of their population affinities. This approach infers *K* distinct genetic clusters, each characterized by a set of allele frequencies at each locus, and estimates the proportion of individuals with ancestry in each cluster. We ran 15 independent simulations of 1,000,000 iterations (following a burn-in period of 100,000) for each value of *K* (Pritchard et al. 2000) between 1 (the expected value if all populations belonged to the same breeding deme) and 21 (the maximum possible number of populations). The admixture model was applied assuming gene flow among populations and allowing for correlated allele frequencies across populations. We performed two different models, assuming the default mode for STRUCTURE that uses only genetic information to learn about population structure, and the LOCPRIOR model that uses sampling locations as prior information to assist the clustering.

Appropriate *K* values were selected using the natural log of Pr(X|K) values reported by STRUCTURE and the ΔK criterion (Evanno et al. 2005) by taking into account the shape of the log-likelihood curve with increasing *K* and variance among estimates in multiple runs. Replicate runs were averaged using CLUMPP v1.1 (Jakobsson and Rosenberg 2007) and plotted in DISTRUCT v1.1 (Rosenberg 2004) to generate graphical displays with the membership of each individual representing the mean membership over the replicate runs.

We also investigated the genetic structure in more detail with a Bayesian model-based clustering algorithm implemented in TESS 2.3.1 (Durand et al. 2009) to incorporate geographic information into the analysis. This model is a spatially explicit clustering model with admixture, in which individual genome proportions are estimated by incorporating spatial trends and spatial autocorrelation into the prior distribution of individual admixture proportions. These proportions are allowed to vary over space, and the variation is decomposed into effects at the regional and local scales. Trend surfaces account for clines in all geographic directions, and autocorrelated residuals account for isolation by distance. The parameters specifying the shape of the clines are also estimated from the data, together with the magnitude of spatial autocorrelation. The model, implemented in a Markov Chain Monte Carlo (MCMC) algorithm, can therefore be used for the simultaneous detection of clines and clusters, through studies of the inferred variation of admixture proportions (Francois and Durand 2010). We ran TESS under the conditional autoregressive (CAR) Gaussian model of admixture with a linear trend surface (Durand *et al*. 2009), with updating of the admixture parameter (α) and the interaction parameter (ρ), which were set to default values of $\alpha = 1$ and $\rho = 0.6$. The algorithm was run with a burn-in period of 20,000 cycles and parameters were estimated with 50,000 additional cycles. We varied the maximal number of clusters (*Kmax*) from 2 to 10, with 20 replicates for each value of *Kmax*. The maximum number of clusters that best fit the data is chosen using the statistical Deviance Information Criterion (DIC). Outputs from TESS analyses using the identified value of *K* were averaged over the 20 runs using the computer program CLUMPP version 1.1 (Jakobsson and Rosenberg 2007; 'greedy' algorithm, 100 random input sequences, G' statistic).

The population clusters identified in STRUCTURE were used in Analysis of Molecular Variance (Amova, Excoffier et al. 1992), implemented in Arlequin v.3.5.2 (Excoffier et al. 2005), to examine the distribution of genetic variation at three hierarchical levels: within populations, among populations within demes, and among demes. At first, we assigned each population to a cluster based on its largest proportion of membership. We then examined hierarchical genetic distribution considering only populations with a mean membership ≥ 70%, and therefore clearly assigned to one genetic deme.

We estimated recent migration rates between localities using the program BAYESASS 3.0 (Wilson and Rannala 2003), which employs a Bayesian MCMC approach to identify migrants or recent descendants of migrants based on transient linkage disequilibrium among multilocus genotypes from different source populations. This program was run following the recommendation of Faubet et al. (2007) concerning convergence issues. We performed 10 separate runs, with different seed numbers, using of 10^7 iterations, with a burn-in of 10^6 and a sampling frequency of 1000. This run length was sufficient for the posterior probability to achieve convergence. The MCMC mixing parameters were set to 0.50, 0.80 and 0.25, for allele frequency, level of inbreeding and migration rate, respectively. These delta values resulted in acceptance ratios between 40% and 60%, in order to maximize log likelihood values (Wilson and Rannala 2003). Migration rates were averaged over the 10 independent runs and compared to average migration rates of randomly permuted data set (generated in GENODIVE) to assess significance. Estimated migration rates were considered significant when the 95% confidence interval (CI) did not overlap with the 95% CI of the randomly permuted data.

Results

Within-population patterns of genetic diversity

Our microsatellite markers were highly polymorphic across populations sampled, except for LVG-210 and LVG-388, which were monomorphic in 3 and 9 populations, respectively (Table S1, Supplementary material). Overall, allele numbers per locus ranged from 3 (locus LVG-388) to 46 (loci Lm_488 and Lm_749). Within populations, the mean number of alleles ranged from 4.5 to 10.6 (Table 1). Across all loci, expected heterozygosity ranged from 0.048 to 0.955, and observed heterozygosity ranged from 0.048 to 1.00 (Table S1, Supplementary material). On average, loci and populations conformed to HWE, with three loci showing deviations, but not at all populations (Lm_521, Tv3Ca19 and Lm_488; Table S1, Supplementary material). Ten populations showed deviation at one locus. However, those loci did not show consistent deviations across all populations; therefore, we assumed that processes causing this nonequilibrium were specific to those populations, and continued to include those loci in subsequent analyses. We detected no linkage disequilibrium between any of the considered 11 loci.

A few populations showed slightly lower levels of diversity (FT, RP, FP). Expected heterozygosity ranged from 0.507 to 0.681 and was lowest for FT population and highest for RR population (Table 1). Across sampled populations, allelic richness ranged from 4.447 to 8.455, with FP population showed the lowest allelic richness, while FER population showed the highest allelic richness (Table 1). F_{IS} estimates were significantly greater than zero in 6 of the 21 demes. F_{IS} values ranged from 0.002 to 0.132 (Table 1). The significant F_{IS} values represent moderate deviations from Hardy-Weinberg expectations, likely due to nonrandom mating within populations.

Among-population patterns of genetic diversity

Pairwise F_{ST} values ranged from 0.001 to 0.239 among all 21 breeding aggregations, with an average F_{ST} = 0.066, and were significantly higher than zero in 202 of 210 cases after sequential Bonferroni correction for multiple comparisons (Table S2, Supplementary material). Most of these values represented low to moderate levels of population differentiation, whereas FT population showed the highest F_{ST} values ranged from 0.119 to 0.239, representing moderate to high levels of differentiation.

The number of migrants (*N*m) between populations, calculated directly from the F_{ST} values (in Table S2, Supplementary material; above diagonal), ranged from 0.797 individuals (FT-FP) to 249.750 individuals per generation (FER-RER). The lowest *N*m estimates calculated from pairwise F_{ST} values ranged from 0.797 to 1.845 for FT population comparisons. Overall, *N*m values suggested some evidence of dispersal among the considered populations, in particular within the clusters identified by the Bayesian analyses.

Genetic distances (F_{ST}) among pairs of all populations were significantly correlated with geographical distance between localities (Mantel test, P = 0.013). However, geographical distance explained less than 14% of the genetic variation (r^2 = 0.137) in the study area; therefore, we did not detect a clear pattern of IBD at this geographical scale (Fig. 2A). The subsequent test for IBD on populations with exclusion of FT, FP and RP populations that we considered as outliers (i.e. those demes showing significant higher F_{ST} values despite similar geographic distance in respect to the others) revealed that there was a high significant correlation with geographical distance (Mantel test, P = 0.001) explaining about 43% of the genetic variation (r^2 = 0.428) in our populations (Fig. 2B), with a clearer detected pattern of IBD.

Then we used pairwise F_{ST} (Table S2, Supplementary material) in a PCoA to investigate the relative position of populations in multidimensional space. The first three principal coordinates (PCo) axes explained 21.84, 20.49 and 13.51% of the genetic variation among our populations, respectively, for a total of 55.84%. Scattergram showed low correlation with geography (Fig. S1, Supplementary material). The strongest signal was represented by FB and RR populations, which were geographically separate from the others, whereas FT population seemed to be fairly isolated from the others, displaced along PCo Axis 1. Most populations showed clustered distribution and mixed with each other in the PCoA multidimensional space. A close analysis of the average F_{ST} values per site revealed two distinct patterns: (i) some populations showed significant higher values irrespective of their geographic isolation (i.e. FT and RP-FP pair; Fig. 3), and (ii) the artificial site (trough) in each site pair showed significant higher values than the natural one (Table 3, Figg. 4A and 4B).

Bayesian estimates of genetic structure

The Bayesian estimates of population structure partially corroborated our distance-based analyses. Alternatively, the model-based clustering method implemented in STRUCTURE suggested that there were either 3 (highest Δ*K*) or 5 (highest likelihood) groups. The model with $K = 5$ (where *K* is the number of genetic population clusters) was substantially better than alternative models. The highest posterior probabilities for *K* varied among multiple runs with the ‘best’ *K* ranging from 5 to 8. Our values of LnProb(data) showed a pattern of incremental increase with increasing *K*; the curve plateaued and continued to increase with larger values of *K*, leading to potential overestimates of the number of populations. Instead, Δ*K* value takes into account the shape of the log likelihood curve. For our data, $\Delta K = 3$ was 17.1 and 42.9 (with and without Loc Prior, respectively), the highest values, whereas the estimates at $\Delta K = 5$ were 5.3 and 6.9 (with and without Loc Prior, respectively). The values for all other possible runs were less than 1.

The plot of $K = 3$ suggested that FB and RR demes grouped into a two-population cluster, whereas there were other two distinct clusters broadly corresponding to north-western and south-eastern geographical sector of the study area (Fig. 3). Mean membership coefficients for all individuals into one of these two demes were generally high, with a mean of 0.821 (SD = 0.167).

The plot of $K = 5$ distinguished the same three clusters, but also identified a cluster composed by a signle population (FT), and another represented by FP and RP populations (Fig. 5). Mean membership coefficients for all individuals into the north-western and south-eastern demes remained generally high, with a mean of 0.796 (SD = 0.160) and 0.751 (SD = 0.235), respectively. A few populations (F23, RIC, FBG, F19 and R19), located on the border of these two clusters, showed membership coefficients less than 70% and thus may be considered as admixed populations (Fig. 5; Fig. 6).

The Bayesian clustering analyses performed by TESS confirmed the results provided by STRUCTURE. Indeed, $K = 5$ was the best clustering option for the dataset, because only a minor decrease in the deviance information criterion (DIC) values was observed at higher K values. DIC values with the microsatellite dataset reached a plateau at $K = 6$, but the inspection of the plotted membership coefficients showed that only five clusters were represented.

Assuming that the genetic clusters inferred from the Bayesian analyses represent panmictic demes, we estimated F_{ST} and calculated an Amova for populations in these genetic clusters. A hierarchical Amova revealed that 86.73% of genetic variation resides within populations, 7.89% is distributed among populations within clusters, and 5.38% of the variance can be explained by differentiation among the five clusters (Table 2). Although the proportion of genetic variation accountable at higher levels is small (5.38%), all fixation indices are statistically significant. The results were similar if we exclude admixed populations (F23, RIC, FBG, F19 and R19; Table 2). Therefore, the genetic structure detected by clustering methods and population-based F_{ST} analyses is mirrored in the Amova. Little genetic variation could be attributed to differences among populations within clusters, suggesting that they represented clusters of populations that were genetically homogeneous. In addition, most of the genetic variation among breeding aggregations at this geographical scale was found at the level of populations. Our Bayesian clustering analyses indicate that many populations within demes have high membership coefficients and therefore belong primarily to one genetic pool; however, some populations and individuals are admixed (Fig. 6), a pattern that presumably results from dispersal among some of our sampled populations. These results were corroborated by the BayesAss analyses showing similar results for contemporary immigration rates into each population (Table S3, Supplementary material). Few pairwise comparisons between localities (5%) detected recent migrations, indicated by values above 0.037 (distinguishable from the values generated by the simulation with no information in the data set). In 20 instances, the migration rate was greater than 0.04, which indicates the occurrence of substantial contemporary gene flow. Migration rates were especially high ($m > 0.1$, which indicates that more than 10% of the population are recent immigrants) in 12 cases. In these cases, the mean number of immigrants falled outside of the confidence intervals expected in cases of insufficient signal in the data. In most of these cases of high gene flow, breeding populations were geographically close to other neighboring populations and thus members of the same genetic deme (i.e. FB-RR; FP-RP; FER-RER; Table S3, Supplementary material; see also Fig. 3). These incidences of migration included emigrations from only seven source populations (RR to FB; F32 to FFA, FE, LE, F35; F29 to FUA; R19 to FUA, F23, F11, RIC, FBG, F19, RT; FP to RP; FER to RER, RT; RER to FER). A close inspection of the five natural/artificial site pairs revealed that the direction of the migration is clearly linked to the hydrology (permanent vs. temporary) of the site rather than its typology (natural vs. artificial); that is: source populations are represented by permanent sites (i.e. those with water available to netws most of the year), whereas populations receiving individuals inhabited temporary sites (i.e. those with presence of water limited to few months throughout the year) (Table 4). When the site pair consisted of two permanent sites (RER-FER), the emigration between is bi-directional. In addition, FER and R19 represent the source demes for RT, a brook that dries out during summer. Most of the detectable recent dispersal events occurred within site pairs but few other migration events involved nearby populations belonging to a same cluster. This would suggest that the geographical scale at which dispersal occurs for *L. v. meridionalis* in rural landscape is usually in the range 0-500 meters (i.e. 400-500 m is the average migration distance of newts sized as *L. vulgaris*, as suggested by Joly et al. 2001) but occasionally can cover larger distances of few kilometres (see Fig. 1).

Discussion

Population pattern of genetic diversity

The genetic differentiation of Italian smooth newt populations in our network of aquatic systems was moderate (average F_{ST} = 0.066). Population genetic studies in amphibians have mainly focused on large geographic scales and consequently reported higher absolute values of population differentiation (Newman and Squire 2001). However, fine-scale studies in urodelans employing microsatellite markers (Jehle et al. 2005; Zamudio and Wieczorek 2007; Purrenhage et al. 2009) found levels of differentiation comparable to our study. Furthermore, the study species exhibits comparable levels of genetic variation to other pond-breeding amphibians (Scribner et al. 2001; Brede and Beebee 2004; Palo et al. 2004). For *L. vulgaris meridionalis*, genetic data on population differentiation are only available from Kalezic and Tucic (1984), who found moderate to high population allozyme differentiation levels (F_{ST} = 0.06 to 0.40) among close populations. However, direct comparison of F-statistics derived from allozymes and microsatellites is somewhat spurious, as the latter tend to give larger genetic distances and lower levels of inbreeding (Scribner et al. 1994). Our populations showed high variations in average F_{ST} values. We found two distinct patterns for F_{ST} variation (i) among demes and (ii) within site pairs: (i) some populations showed significant higher values than the remaining demes that were not consistent with the geographic distribution (i.e. FT, FP and RP); (ii) within the considered site pairs, as a general rule, populations in artificial water bodies (troughs) had significant higher F_{ST} values than those in natural sites, and this pattern was consistent within each site pair.

As for the first pattern, the higher degree of population differentiation in FT and FP-RP pair was discerned from the lower allelic richness and the significantly lower degree of heterozygosity carachterizing these demes. The relatively longer time-span since isolation and higher level of habitat fragmentation that caracherize

FT and FP-RP populations could be the cause of the higher pairwise F_{ST}-values observed. It is likely that in the past (until 1950), the studied area consisted of a unique large area of unfragmented habitats with many natural aquatic systems as potential breeding sites for the Italian smooth newt. In the 1950s habitat loss and fragmentation has been increased because of wide-scale human impact probably isolating FT and FP-RP in landscapes characterized by less favorable habitats. Indeed, the FT population inhabited a dense vegetated area surronded by a highway, several roads, built-up areas and cultivated lands, whereas FP-RP population occupied an open and dry area close to a highway. Such habitat fragmentation may have reduced genetic variability and migration among local populations (Nei 1987; Frankham 1995) making FT and FP-RP more structured and differentiated compared with other populations. Our suppositions are in agreement with previous studies documenting the negative effects of habitat fragmentation on local genetic variability and connectedness in amphibian populations (Reh and Seitz 1990; Hitchings and Beebee 1997; Young and Clarke 2000; Johansson et al. 2005). Linear landscape elements (i.e., roads and cultivated lands), in particular, are known to influence amphibian movements, and hence also the genetic diversity within local populations (Vos and Chardon 1998; Vos et al. 2001; Lesbarreres et al. 2003). Indeed, dispersal distances of Italian smooth newt individuals have been estimated to be 400-500 m (Joly et al. 2001) with a strong negative effect roads and cultivated lands on breeding site occupancy (Razzetti and Bernini 2006). The species' life history may also counteract a possible genetic drift for FT and FP-RP demes related to their geographical and ecological isolation. Since Italian smooth newts become adult in the second-third year of life (Nobili and Accordi 1997), relatively few generations (max. 30) have passed since habitat fragmentation started in the study area. The occurrence of overlapping generations and the possibility of reproductioln strategy involving multiple paternity (Jehle et al. 2007; Rovelli et al. 2015) may also contribute to the maintenance of genetic diversity in such populations. Therefore, the estimate of the number of migrants per generation based on F_{ST} values (Nm) may be an overestimation for the present landscape conditions.

As for the second pattern (F_{ST} variation within site pairs), we hypothesized that the higher values found in artificial sites could reflect the colonization dynamics from a natural site. Indeed, populations inhabiting drinking troughs likely colonized artificial habitats by dispersal from a neighbor natural site (brook, pond, pool). Thus, although the colonization event can date back a maximum 70 years ago (i.e. most drinking troughs in the area were built just after World War II), the amount of estimated genetic differentiation should be related to relatively fast drift processes acting on small-sized populations in small and isolated sites (Allentoft and O'Brien 2010; Noël and Lapointe 2010). We believe that these populations could have very low effective population size at the beginning of the colonization process and experienced intermittently demographic contractions when drinking troughs underwent severe management, allowing for fast genetic drift and subsequent population differentiation (Noël and Lapointe 2010).

We also found a significant correlation between genetic and geographical distance measures. Indeed, the dispersal rate between populations decreased with distance, and this can be measured also over small distances. For an isolation by distance (IBD) relation to establish between populations, dispersal between neighbor breeding sites has to be possible but is restricted, so that populations differentiate from each other with increasing distance (Hutchinson and Templeton 1999). Indeed, we found a significant IBD pattern between populations separated by a maximum distance of 16.7 (FFA-FER) and 17.8 km (FB-FE). Once FT and FP-RP, considered as outliers in terms of genetic but not of geographical distance, were excluded, IBD relationships increased their statistical significance with a higher explained genetic variation. However, this moderate correlation coefficient suggests that further processes other than distance may have structured the genetic composition of populations. According to Bossart and Prowell (1998), FT and FP-RP populations would have not been connected by gene flow showing a relatively higher level of genetic differentiation compared with other populations separated by the same geographic distance.

Although the level of population differentiation is low, the current permeability of the landscape matrix suggests that this genetic structure is unlikely to reflect present day gene flow levels although a definitive conclusion can not be drawn based on the present data. Nevertheless, we feel that our assumption of the genetic structure reflecting an intermediate situation between the historic situation in 1950 and the current landscape is highly plausible. If the time since separation has been insufficient for complete divergence due to drift or to accumulation of mutations, populations will still resemble each other even in the absence of present-day gene flow (Bossart and Prowell 1998).

Population genetic structure

Investigation of genetic structure in our 21 populations of *L. v. meridionalis* revealed five population clusters with a statistically significant level of genetic differentiation. Gene flow seems to be extensive among populations within each cluster determining genetically homogeneous assemblages as indexed also by pairwise population F_{ST} and Nm comparisons, and most genetic variation can be explained by differences within populations. Bayesian estimates of recent migration rate confirmed the existence of a high level of connectivity among the majority of the study natural and artificial breeding systems. Drinking troughs and natural permanent aquatic systems within each cluster exhibited substantial migration rates such that they can be considered nearly panmictic breeding groups.

Three clusters of populations in our sample show similar degrees of cohesiveness concordant with the geographic distribution with nearby populations less genetically differentiated than those farther, thus evidencing a significant positive relationship between differentiation (measured as F_{ST}) and geographical distance (Spear et al. 2005). We estimated low to moderate levels of population differentiation that probably reflect the counterbalance of different effects currently ongoing or acted in the past favoring (i.e. the natural limits to dispersal and population-level processes imposed site fidelity and low vagility; Irwin 2002) or reducing (higher gene flow levels likely occurring when pristine habitats were widespread in the area) genetic differentiation. For example, homing behavior exhibited by the study species influences population genetic structure by reducing gene exchange among local breeding populations (Hedgecock 1978; Kalezic and Tucic 1984). Indeed, the Italian smooth newt during the terrestrial phase is known to prefer shelters and hibernation sites in well-vegetated areas nearby the reproduction sites (usually 10-60 m) (Razzetti and Bernini 2006).

Five populations, even if assigned to a given cluster, showed evidence of substantial mixed genetic ancestry. Such admixed populations, geographically intermediate between the ranges occupied by each cluster, are expected in systems where geographical structure exists but where there are no absolute geographical barriers to dispersal (Zamudio and Wieczorek 2007). Our study area likely conforms to this landscape scenario. Indeed, the Tolfa Mountains represent one of the highest wilderness areas in Italy where the extensive rural and husbandry activities are still carried out following traditional practices and are integrated with large patches of natural habitats (Gimona 1999). Thus, it is likely that geographic barriers, even if occur, do not severely limit the overall individual exchange among populations.

Two clusters that have drifted apart showed genotypic frequencies discordant with the geographic distribution. Landscape features alone or in combination with topographical differences likely have limited dispersal of the individuals, thus producing the estimated high differentiation and isolation of these two clusters. However, without a thorough landscape analysis (identification of actual and past barriers and quantification of frictions to newt movements; Rothermel and Semlitsch 2002), any inference on the role of landscape features on genetic structure and population dynamics remain just plausible. Moreover, we cannot exclude that the observed genetic patterns could have been established prior to habitat modifications (Zamudio and Wieczorek 2007).

Our estimated migration rates showed that current migration between populations from different sites rarely occur (only in 20 cases out 400 possible population combinations - 5%) and has been detected only within clusters. Interestingly, nearly all the migration instances consisted in unidirectional events with only seven populations acting as source of individuals migrating towards other demes. A close inspection of the 20 migration instances revealed a pattern where the site hydrology (permament vs. temporary) rather than the typology (natural vs. artificial) drove the migration direction. As expected, natural permanent sites acted as population sources for migrants. However, also small artificial sites can provide migrants to other demes but only if they guarantee water availability permanently. Long and developed brooks (i.e. RT) that periodically undergo severe drought events do not serve as source but receive individuals from natural and artificial source sites.

From *N*m estimates we could grossly estimate past migration rates that are consistent with the pattern uncovered by BayesAss (i.e. migration events confined to population belonging to same clusters) but apparently occurred at higher frequency and involved a higher number of populations. This comparison has to be considered spurious (see above our discussion on the limitation of inferences on migration based on *N*m values) but evidences a possible reduction of gene flow among populations in the recent years due to environmental changes and habitat alterations subsequent to two main recognized land cover changes occurred from 1950 to nowadays in the area: (i) land abandonment, and (ii) forest loss due to conversion to pastures and shrublands and to intensive grazing, fires, climate aridity, and increasing human pressure (Salvati et al. 2017).

Our data indicated high connectivity among drinking troughs and between drinking troughs and natural permanent aquatic systems belonging to a given cluster. Such detected connectivity suggests that both artificial and natural aquatic systems may constitute metapopulations in which both recolonization events subsequent to local extinctions and natural restocking after severe demographic contractions are likely to occur.

Conclusions

Our study revealed that population differentiation is evident in *L. vulgaris meridionalis* even at a fine scale. Populations are genetically distinct from each other maintaining their own genetic identity. However, although they can be clustered into larger genetic groups based on their genetic similarities, such clusters seemed not to be completely panmictic, despite the presence of admixed individuals in some populations. For a more functional connectivity measure, research on *L. vulgaris meridionalis* movement behavior within landscapes is needed (Opdam et al. 2003). In order to get a better insight into what extent genetic differentiation is related to reduce dispersal, it would be desirable to compare Italian smooth newt genetic structure in landscapes with different degrees of habitat fragmentation (Arens et al. 2007).

As far as we are aware of, the effects of small artificial permanent aquatic water bodies as surrogated of natural habitats on amphibian population genetic diversity have not been previously assessed, and our findings on well structured populations inhabiting the studied network of drinking troughs are noteworthy from a conservation perspective. Indeed, if populations encompass more than a single breeding site, drift and local loss of genetic diversity would be diminished (Arens et al. 2007). The integration of artificial aquatic habitats in the natural network consisting of ponds, brooks, and pools is not only just an extension of pre-existing wetlands but represents a significant and irrepleceable improvement of network functionality in the current scenario of progressive and incessant process of aquatic habitat loss. The restored functionality of the natural network of wetlands by the integration of drinking troughs in the rural/natural landscape is crucial for amphibian conservation as the ensemble of artificial and natural permanent aquatic system provide breeding sites and safe migration routes (Johansson et al. 2005).

Due to the disappearance of most pristine aquatic habitats subsequent to human and natural envrinmental changes, drinking troughs may represent a last testimony of the original genetic diversity and structure of natural populations that is mandatory to be preserved.

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Table 1 Details of study populations. Number of smooth newts genotyped (*N*), allele number (*N*a), allelic richness (*A*r), private alleles (*P*a), mean observed (H_O) and expected (H_E) heterozygosities for the eleven microsatellite loci, and F_{IS} with *P* value (*significantly greater than zero in 6 of the 21 populations, using a randomization test with 10.100 randomizations) for each of the sampled *Lissotriton vulgaris meridionalis* breeding populations

Pop Code	***N***	***Na***	***Ar***	***Pa***	***Ho***	***He***	***Fis* (P value)**
FB	17	6.000 (0.915)	5.888	0.455 (0.207)	0.613 (0.085)	0.617 (0.078)	0.037 (0.281)
RR	33	7.818 (1.340)	6.760	0.364 (0.364)	0.629 (0.083)	0.681 (0.070)	0.092 (0.002)*
FT	20	4.727 (0.764)	4.552	0.182 (0.122)	0.515 (0.102)	0.507 (0.086)	0.012 (0.507)
FFA	20	6.909 (1.443)	6.573	0.091 (0.091)	0.591 (0.098)	0.630 (0.100)	0.088 (0.023)
F32	20	7.364 (1.429)	6.924	0.091 (0.091)	0.646 (0.089)	0.654 (0.082)	0.037 (0.199)
FE	20	7.727 (1.613)	7.164	0.091 (0.091)	0.559 (0.097)	0.619 (0.093)	0.122 (0.001)*
LE	20	8.091 (1.729)	7.614	0.091 (0.091)	0.670 (0.099)	0.662 (0.087)	0.014 (0.562)
F35	20	7.909 (1.575)	7.463	0.000 (0.000)	0.625 (0.075)	0.656 (0.069)	0.074 (0.038)
F29	20	5.818 (1.069)	5.608	0.000 (0.000)	0.527 (0.077)	0.583 (0.079)	0.122 (0.004)*
FUA	20	7.818 (1.747)	7.386	0.000 (0.000)	0.555 (0.089)	0.621 (0.090)	0.132 (0.0001)*
F23	20	8.455 (1.755)	7.854	0.273 (0.141)	0.623 (0.096)	0.637 (0.099)	0.048 (0.119)
F11	20	7.545 (1.592)	7.026	0.000 (0.000)	0.541 (0.086)	0.600 (0.091)	0.123 (0.002)*
RIC	17	8.273 (1.722)	8.120	0.182 (0.122)	0.602 (0.099)	0.647 (0.093)	0.100 (0.008)
FBG	20	7.364 (1.403)	7.008	0.000 (0.000)	0.627 (0.096)	0.636 (0.094)	0.040 (0.160)
F19	20	6.909 (1.609)	6.537	0.091 (0.091)	0.573 (0.103)	0.565 (0.102)	0.012 (0.409)
R19	20	7.818 (1.710)	7.364	0.000 (0.000)	0.568 (0.110)	0.607 (0.101)	0.089 (0.013)
RP	21	5.364 (1.106)	4.983	0.000 (0.000)	0.550 (0.106)	0.547 (0.103)	0.019 (0.357)
FP	20	4.545 (0.608)	4.447	0.091 (0.091)	0.573 (0.091)	0.566 (0.081)	0.014 (0.421)
RT	30	9.545 (2.121)	7.861	0.364 (0.203)	0.602 (0.094)	0.652 (0.097)	0.095 (0.001)*
FER	34	10.636 (2.394)	8.455	0.364 (0.279)	0.668 (0.095)	0.659 (0.095)	0.002 (0.556)
RER	32	9.727 (2.253)	8.013	0.182 (0.182)	0.627 (0.105)	0.654 (0.097)	0.056 (0.034)

Table 2 Results of hierarchical Amova comparing genetic variation within breeding populations, among breeding populations within the five clusters, and among clusters. Significance was tested against a null distribution of 10000 random permutations. The test was performed twice, including and excluding admixed populations. Significant *P* values are indicated with an asterisk

Source of variation	**d.f.**	**Sum of square**	**Percentage of variation**	***P* value**
Including admixed populations				
Within populations	464	1524.000	86.73	**< 0.001***
Among populations within clusters	20	1781.911	7.89	**< 0.001***
Among clusters	4	143.065	5.38	**< 0.001***
Excluding admixed populations				
Within populations	367	1205.000	85.78	**< 0.001***
Among populations within clusters	15	1402.708	7.73	**< 0.001***
Among clusters	4	144.574	6.49	**< 0.001***

Table 3 General Linear Model performed on F_{ST} values in eight demes representing four site pairs (A=RR-FB, B=R19-F19, C=RP-FP, D=RER-FER). There was a clear effect of site typology (TYPE: natural vs. artificial) with artificial sites presenting higher values, and each pair showed different F_{ST} values

Effect	SS	Df	F	p
Intercept	0.889	1	759.304	0
PAIR	0.079	3	22.486	0
TYPE	0.014	1	11.997	0.001
PAIR*TYPE	0.002	3	0.475	0.700
Error	0.178	152		

Table 4 Description of the natural/artificial site pairs, their hydrology and direction of the migration among newt populations at the study area. The arrows indicate the direction of gene flow between populations of each pair. Abbreviation: p=permanent; t=temporary; MD=migration direction

Pair	Site pair				Other sites reciving newts	
	Natural	MD	Artificial	MD	Natural	Artificial
RR-FB	RR(p)	→	FB(t)			
RER-FER	RER(p)	↔	FER(p)	→	RT(t)	
RP-FP	RP(t)	←	FP(p)			
R19-F19	R19(p)	→	F19(t)	→	RT(t)	FUA(p)-F23(p)-F11(p)-RIC(t)-FBG(p)
LE-F32	LE(t)	←	F32(p)	→		FE(t)- FFA(p)-F35(p)

Fig. 1 Topographic map of Tolfa Mountains, with localities for 21 *Lissotriton vulgaris meridionalis* populations sampled for this study. Each colored ID corresponds to the assigned cluster

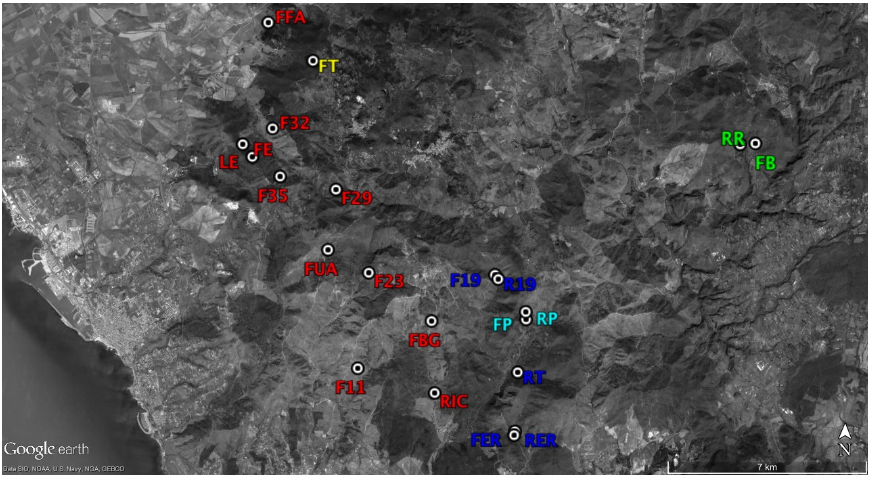
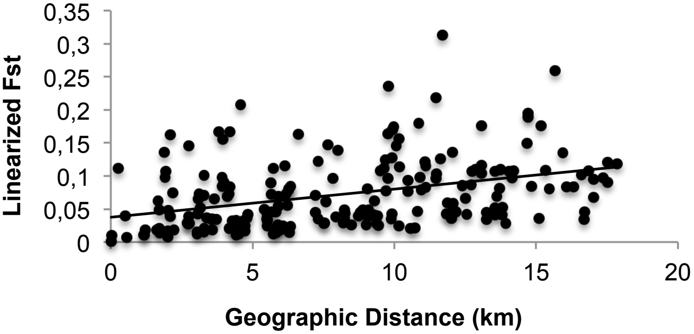


Fig. 2 Relationship between pairwise geographic and genetic distances (linearized F_{ST}) across *Lissotriton vulgaris meridionalis* populations. Mantel tests reveal a significant correlation in both cases. **A**: all sampled populations; **B**: populations with exclusion of potential outliers

A



B

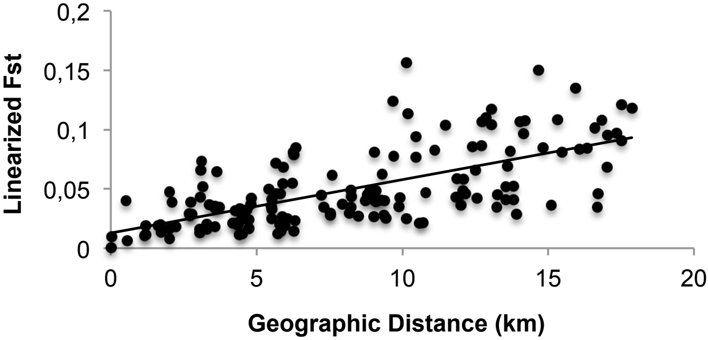


Figure 3. Average F_{ST} values in the sampled sites. Points indicate means; bars indicate standard errors

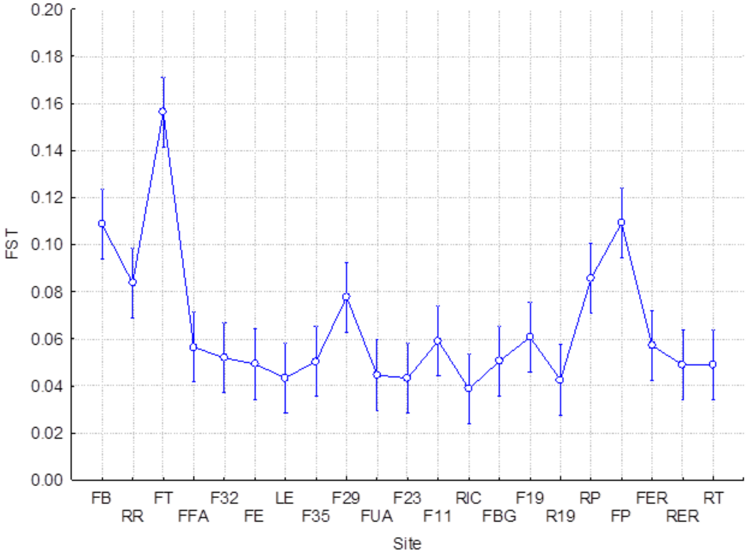
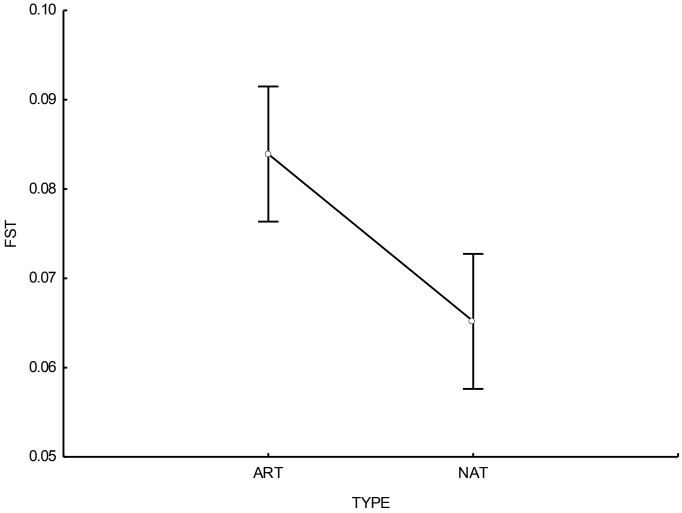


Figure **4.** A: Comparison of F_{ST} values between artificial and natural sites belonging to site pairs; B: interaction effect between PAIR (site pair) and TYPE (artificial vs. natural) on F_{ST} values. Pair codes: A=RR-FB, B=R19-F19, C=RP-FP, D=RER-FER

A



B

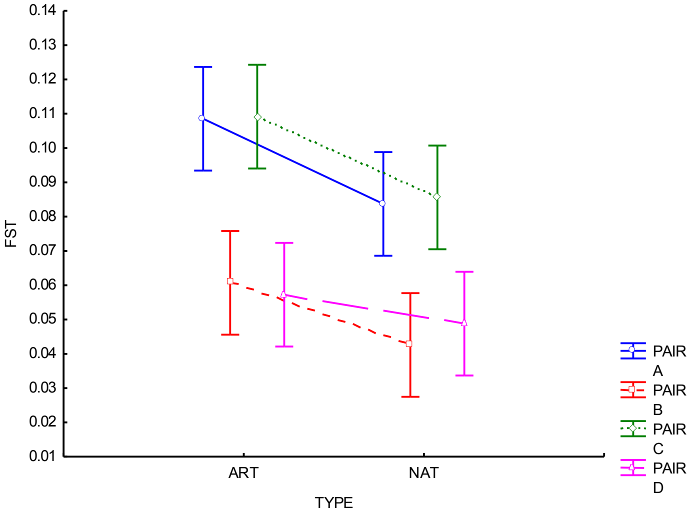


Fig. 5 Population structure inferred by Bayesian assignment of 464 individuals of *Lissotriton vulgaris meridionalis* performed by STRUCTURE software. Italian smooth newt populations in Tolfa Mountains can be assigned to five clusters of populations

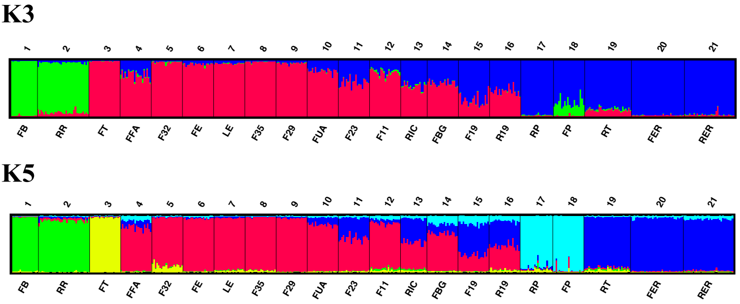
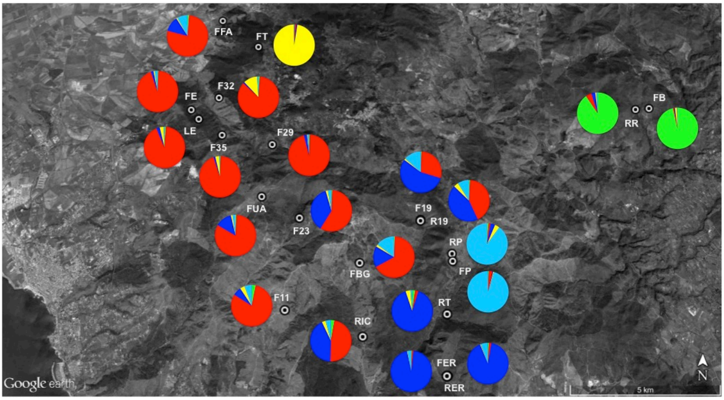


Fig. 6 Topographic map of Tolfa Mountains, with localities for 21 *Lissotriton vulgaris meridionalis* populations sampled for this study. Pie charts describe the percentage membership to each cluster for *K*=5



FINAL CONCLUSIONS

Amphibians are the vertebrate group experiencing the steeper global population decline and species loss (Stuart et al. 2004). For Mediterranean amphibians, the major threats are represented by habitat loss, alteration and fragmentation caused by agricultural intensification (Stuart et al. 2004; Cushman 2006). Aquatic habitats, representing reproductive sites for many amphibian species, deserve a particular attention since their reduction in number and quality is quite alarming (Stevens et al. 2002). It has been suggested that artificial aquatic sites may represent an important contribution to the potential breeding habitats for amphibians, especially in human modified environments (Knutson et al. 2004; Garcia-Gonzalez and Garcia-Vazquez 2011; Hartel et al. 2014). Indeed, the ability of amphibians to colonize successfully such artificial aquatic sites is well recognized (Petranka et al. 2003; Rannap et al. 2009; Lannoo 2014). However, few studies have focused on small artificial sites extremely different in shape and size in comparison to natural ones (e.g. tanks, drinking troughs and reservoirs with water surface < 10 m2) (Romano et al. 2014) in favor of those simulating natural ponds (e.g. man-made ponds with smooth shorelines and aquatic vegetation) (Contreras et al. 2009; Knutson et al. 2004).

The present Ph.D. study highlighted that even smaller and completely artificial aquatic habitats, linked to traditional farming practices, provide irreplaceable breeding sites for amphibians and an essential contribution to their conservation. Indeed, drinking troughs, associated with the Maremmana cow breeding, provided suitable alternatives to pristine habitats for the reproduction, feeding and sheltering of *L. vulgaris meridionalis* in our study site. We therefore provided robust evidence that the presence of extensive cattle breeding may have positive effects on amphibian ecology and survival in contrast to previous studies (Knutson et al. 2004; Braun 2006). Livestock wading and defecating, in fact, are known to spoil water quality (i.e., increasing levels of nitrogen and phosphorus, and decreasing oxygen concentrations) and modify aquatic and emergent vegetation in natural and artificial ponds resulting in an adverse environment for amphibian eggs and tadpoles (Knutson et al. 2004).

To date, the persistence of drinking troughs is endangered by land reclamation, urbanisation and the abandonment of traditional farm practices in favor of modern agriculture and intensive farming (Romano et al. 2014). Thus, although preventing the modernization of agriculture is not possible and would be undesirable from a socioeconomic viewpoint, the identification and protection of such key sites may be crucial for amphibian survival (Garcia-Gonzalez and Garcia-Vazquez 2011), especially in a period that is witnessing major changes in local landscapes, due to land use changes, land abandonment, and climate change (de Chazal and Rounsevell 2009).

To help stop amphibian decline and for the correct management and conservation of amphibian species, a deep knowledge of their local population dynamics and their genetic structure is urgently required (Purrenhage et al. 2009). With increasing anthropogenic habitat modification it is particularly important to understand the genetic consequences of changes in landscape composition and configuration, even at a small geographic scale (Johanet et al. 2009). This issue highlights a special need for the application of conservation genetics to amphibian populations, and the development of suitable molecular markers (Jehle and Arntzen 2002). Microsatellites have been used successfully in studies of amphibian effective population sizes and structures, dispersal and migration patterns (Beebee 2005). However microsatellites are still under-represented in population and conservation genetics (Jehle and Arntzen 2002) since the cloning of microsatellite loci requires a time- and money-consuming endeavour (Steinfartz et al. 2004) because of amphibian large genome sizes and low numbers of potentially amplifiable loci (Hendrix et al. 2010; Hauswaldt et al. 2008). Moreover, the cross-amplification of microsatellite loci in amphibian congeneric species often shows unexpected low rates of success because of their large genome sizes (Garner 2002), and/or the taxonomy that is inconsistent with phylogenetic relationships among taxa (Primmer and Merilä 2002). The present Ph.D. project allows to optimize a panel of microsatellite markers (previously developed and tested for cross-species amplification in other *Lissotriton* species-subspecies) suitable for assessing Italian smooth newt population structure and genetic diversity across multi-scale levels from meta-populations to full geographical range. For the Italian smooth newt, no microsatellite loci have been reported thus far and their development will be an important contribution to better understand the ecology and evolution of the species helping to assist its effective management and future conservation activities.

Fragmentation of contiguous habitat by agriculture and urban development can create barriers to amphibian dispersal (Vos et al. 2001; Johansson et al. 2005) resulting in isolated subpopulations more subject to increased risk of local extinction (Frankham 2005; O'Grady et al. 2006), and/or less likely to be recolonized by dispersers from neighboring subpopulations (Purrenhage et al. 2009). However, landscape features (i.e. vernal pools, drainage, ditches, and streams) and artificial facilities (e.g. tanks, drinking troughs and reservoirs) may act as stopovers during dispersal events, thereby facilitating dispersal by reducing effective inter aquatic systems distances (Gibbs 1998; Pope et al. 2000). The importance of artificial facilities, generally associated with traditional agriculture and cattle watering, for amphibians feeding and reproduction has been highlighted by many studies in the Mediterranean (Romano et al. 2014). However, there are no studies on their conservation genetic importance and their potential stepping-stone function in dispersal processes and gene flow maintenance. The present Ph.D. study revealed the importance of drinking troughs on Italian smooth newt population connectivity within a patchy system interested by infrastructural and agricultural development. Drinking troughs supported structured populations connected with neighboring natural and/or artificial aquatic systems with a degree of cohesiveness concordant with the geographic distribution. Such detected connectivity suggests that both artificial and natural aquatic systems may constitute a metapopulation in which recolonization events subsequent to local extinctions are highly likely in our region. Studies estimating the spatial scale of dispersal among populations, in addition to basic demographic research, are needed in order to provide data for effective management and conservation decisions (Purrenhage et al. 2009). Indeed, the determination of genetic relationships among populations could be of assistance to the formulation of management practices, including identifying areas of high conservation priority, revealing dispersal corridors among populations, detecting severe population declines and identifying individuals and/or populations fundamental for augmentation/translocation or reintroduction programmes (Mace et al. 1996).

In conclusion, the present Ph.D. research seems to be particularly valuable since it attempts to recognize firstly the ecological and genetic value of drinking troughs for amphibian conservation filling a gap of knowledge arising from the lack of empirical data on amphibians in such small artificial habitats (but see Garcia-Gonzalez and Garcia-Vazquez 2011) that may have been neglected in research and policy in favor of lakes, rivers and streams (Fuentes-Rodrìguez et al. 2013).

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