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SUMMARY (Italian):

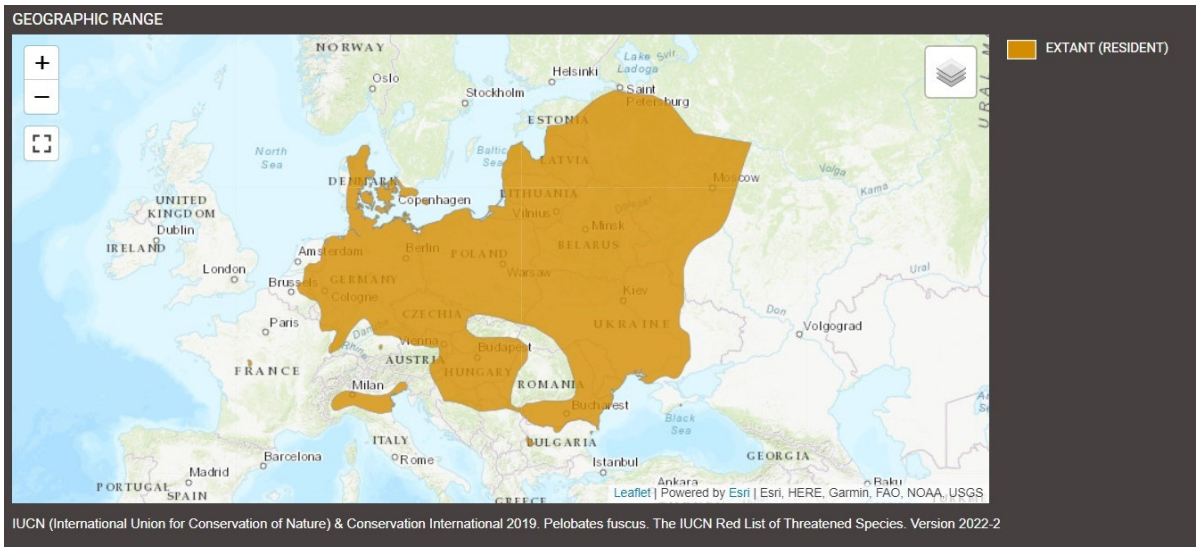
Il pelobate fosco, *Pelobates fuscus* (Laurenti, 1768), è un anfibio anuro distribuito ampiamente nell'Europa centro-orientale. La specie è presente storicamente anche nel nord Italia, soprattutto in aree planiziali come la pianura Padana, in habitat adatti allo stile di vita fossorio e la riproduzione in stagni temporanei. A causa però di alterazioni del territorio, agricoltura intensiva e presenza di ittiofauna, le popolazioni italiane sono in declino. Da alcuni anni il pelobate è quindi oggetto di iniziative di conservazione nell'ambito di progetti LIFE e WWF. In Veneto, dopo la scoperta di una popolazione relitta nel cordone dunale di Porto Caleri (Rosolina, RO), è stato avviato un progetto di reintroduzione di esemplari, a partire da due ovature fatte schiudere in condizioni controllate, nella Riserva Naturale Integrale Bosco Nordio (Chioggia, VE), coordinato da "Veneto Agricoltura".

L'obiettivo di questo lavoro è quello di eseguire, per la prima volta, una caratterizzazione genetica delle due popolazioni, quella reintrodotta e quella sorgente, così da ricavare informazioni utili alla loro gestione futura. A questo scopo è stato caratterizzato il citocromo b mitocondriale e sono stati isolati ed applicati marcatori microsatellite. Al fine di inquadrare le due popolazioni in un contesto più ampio, sono stati analizzati anche campioni provenienti da altre aree geografiche. I risultati del sequenziamento ed analisi mitocondriale hanno permesso di evidenziare la formazione di un gruppo, costituito da individui di Bosco Nordio e Porto Caleri, geneticamente isolato, sia dal resto d'Italia che da popolazioni extra-italiane. Le analisi dei loci microsatellite hanno consentito lo studio della variabilità genetica all'interno delle diverse popolazioni e del differenziamento tra queste. I risultati supportano l'ipotesi di isolamento e un possibile effetto "collo di bottiglia" nella popolazione di Bosco Nordio, con conseguente perdita di diversità allelica, suggerendo una incompleta rappresentazione della popolazione sorgente nel processo di reintroduzione. I risultati ottenuti, sebbene preliminari, forniscono interessanti spunti per una gestione informata delle popolazioni d'interesse.

1. INTRODUCTION

1.1 PELOBATES FUSCUS SPECIES

Pelobates fuscus (Laurenti, 1768) is an anuran amphibian belonging to the family Pelobatidae, which has a Nearctic-Palearctic distribution. Specifically, this family includes the genus *Pelobates*, which geographically ranges between Europe, western Asia, and the extreme regions of north-western Africa. The genus *Pelobates* is represented by 4 species: *Pelobates cultripes*, *Pelobates varaldi*, *Pelobates syriacus* and *Pelobates fuscus*. This last is widespread in Eurasia and includes the subspecies *Pelobates fuscus fuscus* and *Pelobates fuscus insubricus*. The first has a very broad geographic range, from north-eastern France to the Urals, Caucasus, and Kyrgyz steppes in the east; from the south of Sweden to the northern Balkans and the eastern shore of the Caspian Sea regarding the north-south axis. In contrast, *Pelobates fuscus insubricus* is endemic to northern Italy (Andreone, 2001).



(**Fig.1**; *Pelobates fuscus* species geographic range, https://www.iucnredlist.org/)

The common name of the species, *spadefoot toad*, derives from a specific anatomical and behavioral feature. This amphibian is characterized by a typically fossorial and elusive life and is in fact used to inhabit sandy areas, where it burrows during the day, thanks to metatarsal tubercles of its hind limbs that enable it to dig efficiently. European individuals show a specific preferential habitat, which is restricted to friable soils. During breeding periods, however, individuals emerge

from burrows to reproduce within permanent or temporary ponds. From the spawn hatch tadpoles that usually remain in the larval stage for 2-4 months.

Both because of the special living requirements of this species and the amphibians' ectothermy that makes them adapted to specific climates, the population dynamics of the spadefoot toad are strongly influenced by environmental changes. In particular, the current population structure and status was mainly determined by fluctuations of paleoenvironmental conditions (Eggert et al., 2006).

Pelobates fuscus species is considered to be divided into two different lineages, in fact a study done by sequencing the mitochondrial cyrochrome b gene by Crottini et al. (2007) supported the results of Borkin et al. (2001, 2003, 2004) and Khalthurin et al. (2003) regarding the existence of a "western" and an "eastern" lineage. Borkin et al. (2001) considered the two lineages as two different taxonomic unities because they had similar levels of within-population variation but different genome size. In fact, since the cellular DNA intraspecific variation is typically low in amphibians, individuals are conspecifics when they have fundamentally the same DNA amount. However, the study by Crottini et al. (2007) assume that the low levels of molecular divergence between both genome types (western and eastern), and the evidence for introgression do not allow the two lineages to be assigned the species status. Besides this, a study by Litvinchuk et al. (2013) suggested the independence of the two groups as distinct evolutionary species named *P. fuscus* (common spadefoot toad) and *P. vespertinus* (Pallas's spadefoot toad). This theory was subsequently supported in a study by Dufresnes et al. (2019) which provide evidence that the *fuscus* and *vespertinus* lineages correspond to distinct species.

1.2. ITALIAN POPULATIONS

This work focused on *Pelobates fuscus insubricus* (Cornalia 1873), long considered as a separate subspecies inhabiting the Po Plain in northern Italy (Andreone et al., 1993). Later, thanks to a mitochondrial DNA variation analysis, it has been shown that the subspecies formed a lineage within the western *P. f. fuscus* (Crottini et al., 2007). In addition, data from the study by Litvinchuk et al. (2013) based on allozyme approach, genome content and mtDNA through which the genetic divergence from *P.f. fuscus* was measured, do not support the validity of this subspecies name, but includes it within it.

Apart from this, the spadefoot toad inhabiting the Po Valley has a typical morphology of a fossorial animal, is 60 to 90 millimeters long and has relatively short limbs characterized by developed metatarsal spurs. In adults, a small transverse gibbosity can be observed at the top of the head. Male individuals have

no vocal sac and as a peculiar trait exhibit smooth oval glandular formation on the arm, and colorless pearl-shaped outgrowths on the surface of the forearm and on the fingers of the hands. In addition, two different color varieties can be found: *maculate* and *labo-vittata*. The eggs (from 1200 to 3400) are laid together and arranged irregularly in a transparent, gelatinous bead often visible as anchored in the background vegetation. Larvae range from 100 to 120 millimeters in length and vary in coloration depending on the stage of development.

As has already been mentioned, *Pelobates fuscus* leads a predominantly fossorial life, but during spring rains, adults leave their overwintering quarters to travel to breeding sites, preferably temporary bodies of water. This is also because these sites will surely be devoid of ichthyofauna, capable of exerting strong predatory pressure on the species (Andreone, 2001).

A distinguishing feature of the species is also given by the richness of the vocal repertoire. Both males and females emit vocalizations to attract the partner during the breeding season by also performing duets. Since individuals move into the aquatic environment during this period and mating usually occurs at night, thus without light, males and females use low-frequency underwater vocalizations to attract each other. In addition to vocal emissions for reproductive purposes, distress calls are also found but in the terrestrial environment (Andreone & Piazza, 1990).

(**Fig.2**; Adult individual, https://ente.parcoticino.it/)

(**Fig.3**; *Pelobates* spawn anchored in the vegetation, https://www.lifeinsubricus.eu/)

On the Italian territory, the species was first described by Cornalia in 1873, found in the rice fields of Mirasole (Milan), later it was described in 1888 by Héron-Royer under the name *Pelobates latifrons* after specimens were found in the Turin area. These were followed by many reports for the species concerning Piedmont, Emilia Romagna, Veneto and Friuli Venezia Giulia. The first record for the coastal areas of the Po Valley was registered near Ravenna in 1969 (Boldreghini, 1969). The best-known populations are currently found along the moraine elevations of Piedmont and Lombardy and in the lower plains, but populations no longer reported were even located in areas close to urban centers, such as Vanchiglia (Turin),

Mirasole and Porta Vicentina (Milan), Bologna, Bergamo and Brescia (Andreone & Crottini, 2007).

A work by Andreone et al. (2004) reviewed the distribution of *Pelobates fuscus* in the north of Italy, by comparing specimens' findings before and after 1973. Northern Italy was divided in three large areas formed by all the localities where samples of the species have been found, in particular: (1) Piedmont, (2) Lombardy + Emilia Romagna + Switzerland (Canton Ticino) and (3) Veneto + Friuli-Venezia Giulia. What the authors did was simply to put in parallel historical findings (before 1973) and contemporary findings (after 1973). For the three geographic areas the old findings were respectively 11, 22 and 10. Differently, new findings were respectively 15, 20, and 5. The sites that were not reconfirmed, were considered as lost or destroyed, like in Canton Ticino where the population were regarded as extinct. Therefore, results showed that the Piedmont area lost 40% of sites, Lombardy, Emilia Romagna and Switzerland lost 50% while Veneto and Friuli Venezia Giulia lost the large percentage of sites, that was the 66.7%. The authors support the fact that the species is in obvious decline, caused mainly by alteration of the aquatic breeding sites and terrestrial habitats. The causes can be attributed to human activities, in fact, the species is negatively affected by urbanization, intensive agriculture, modifications in forests and ponds and introduction of allochthonous species. In the Po Plain, the effect of environmental changes is quite pronounced, and this brings certain consequences to *Pelobates fuscus* individuals. In addition, natural fluctuations in time, the complexity to monitor the same populations over long-term periods and of course the natural elusive and hidden life of the animal, can explain the decrease in findings. The study also aims to highlight the lack of sufficient research regarding the species.

For all these reasons, the species has been classified by the IUCN as "Endangered" within the Italian Red Lists and awareness campaigns and conservation projects have been promoted (Andreone et al., 2004).

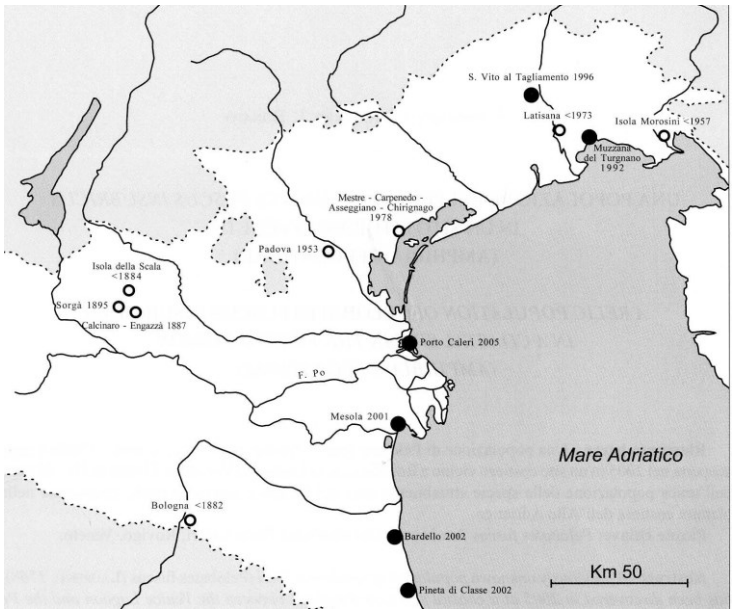
After the Habitat Directive 92/43/CEE of 1992 relative to the conservation of natural and semi-natural habitats and wild flora and fauna, it was possible to benefit from available EU fundings to develop two LIFE Natura projects. The first one was sponsored by WWF Italia and its name was "LIFE IT1110035, *Azioni urgenti per la conservazione del Pelobates f. insubricus*" while the second one was "LIFE00 NAT/IT/007233 – *Progetto Pelobates nel Parco Naturale della Valle del Ticino Piemonte*" (Andreone & Crottini, 2007). A more recent LIFE Natura project (2020 – 2026) was developed in Piedmont and Lombardy, called "LIFE19-NAT/IT/000883 *Insubricus*" and supported by European Climate, Infrastructure and Environment Executive Agency (CINEA) ex-EASME, within the European funding program LIFE 2014-2020 Nature and Biodiversity. The project includes interventions aimed at improving conservation actions for the habitat and the

Pelobates fuscus species living within it, so as to be able to grow populations in the future (https://www.lifeinsubricus.eu/).

1.3. VENETIAN POPULATIONS

In Veneto region, were historically present and documented several sites where *Pelobates fuscus* species was located. In Verona at Isola della Scala, Sorgà, Engazza (Salizzole) and Calcinaro (Nogara) at the end of 1800; in Padua between the end of 1800 and mid-1900; and in Chirignago (Venice) between 1885 and 1920. Other reports in locations close to the latter would have occurred later but not documented and confirmed.

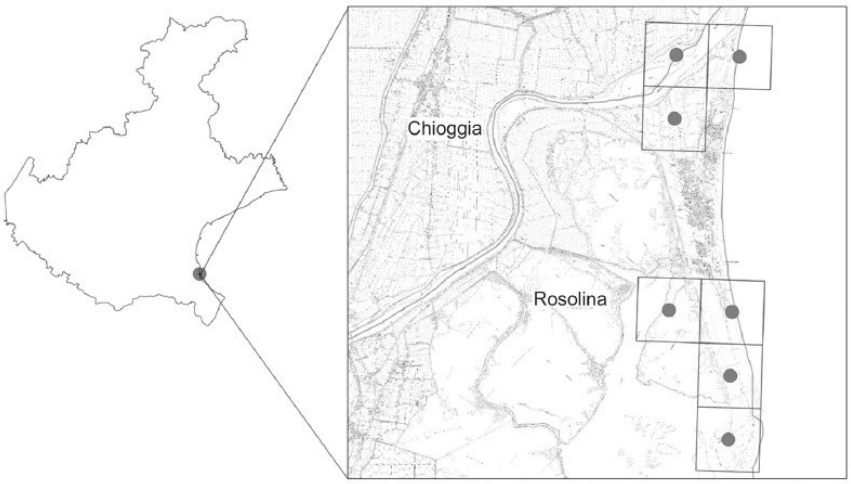
More recently, in 2005, the presence of a population was documented in Porto Caleri, which is part of the municipality of Rosolina in the province of Rovigo. The individuals were found between May and November, always in the first part of the night. It was possible to observe them moving actively on the land. The soil of this area is mainly sandy, particularly suitable for the animal's lifestyle, placed between the sea and the lagoon valleys. The zone is characterized by the presence of dune pioneer vegetation and woods, moreover, it's possible to find lowlands with temporary or perennial ponds. This was the first record of the species in Veneto since the 70's, indeed, it was probably a relict species unknown for long time (Boschetti et al., 2006).



(**Fig.4**; Sites of occurrence of the species in the Veneto region. Black circle = present in the year of the study, white circle = extinct, Boschetti et al., 2006)

A few years later, in 2009, a neo-metamorphosed individual was found in the locality of Porto Fossone (Rosolina), near the mouth of the Adige River (Boschetti et al., 2011).

A study by Bedin & Richard (2016) conducted a survey of the actual distribution of the species in the coastal area of the municipality of Rosolina, through night surveys conducted from 2006 until 2014. The collections of data were distributed between March and October, and the results showed a total of 71 individuals observed in the Porto Caleri area, that is south of Rosolina, and 70 north of the municipality, namely near the mouth of the Adige River. The specimens identified were divided into age classes: juveniles (less than 1 year old) and adults/subadults (greater than or equal to 1 year old). The work showed the presence of two apparent separate populations in the northernmost and southernmost sectors of the littoral, and this appears to be related to the presence of stable dulcequicultural wetlands, suitable as breeding sites for *Pelobates fuscus.*



(**Fig.5**; Sites of occurrence of specimens between Rosolina and the mouth of the Adige River, Bedin & Richard, 2016)

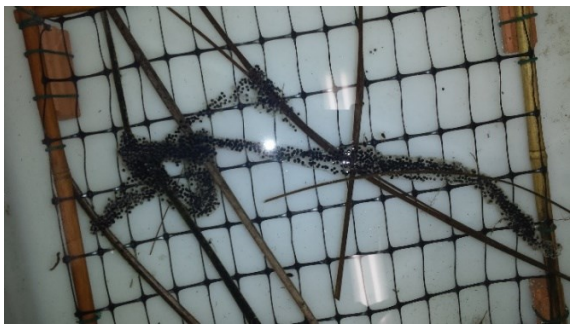
1.4. SPECIES REINTRODUCTION PROJECT IN BOSCO NORDIO NATURAL RESERVE

Thanks to Regional Council Resolution No. 69 of Dec. 13, 2006, a project directed by the "Azienda Regionale Veneto Agricoltura" was activated for the reintroduction of *Pelobates fuscus* within the Bosco Nordio Integral Natural Reserve (Chioggia, VE).

The Reserve was considered particularly suitable for the reintroduction for several reasons. First, because of its geographical location, since it is close (about 6 kilometers) to both Porto Caleri and Porto Fossone; moreover, it is in close proximity to the Adige River, which can function as a passage corridor for the species. Secondly, the environmental type is perfect for the animal's life since it is still a dune system with sandy soil and thermophilic forest. Also favoring the project is the economic-legal side, thanks to the monetary resources that Veneto Agricoltura receives from the European Community and regional funding such as this. In addition, actions designed to benefit amphibians have been included in the park's Management Plan, as well as being itself a Site of Community Importance and a Special Protection Area, pursuant to Directives 92/43/EEC "Habitat" and 79/409/EEC "Birds." An additional resource of the Reserve is nature education aimed at understanding the area.

(**Fig.6**; RNI Bosco Nordio map, https://bosconordio.venetoagricoltura.org/)

The reintroduction process started in 2008, when two spawn were taken from a draining pond in Porto Caleri. These were hatched inside tanks and then reared in a protected environment, and subsequently 989 larvae and 37 neo-metamorphosates were returned to their original location, while 611 larvae and 68 neo-metamorphosates were released in a breeding area in Bosco Nordio, created within a fenced area with a pond inside. In this way, the specimens could be controlled and protected from predators.




(**Fig.7**; *Pelobates fuscus* spawn. Fig.8; Neometamorphosate individual, J.Richard)

This organizational structure of activities maximized the survival rate of the individuals, so much so that after the first singing males were heard in 2010, and after the first egg-laying in 2011, they began releasing animals at two sites in the territory of the Natural Reserve, in or near specially made ponds. Population monitoring gave positive results starting in 2012, then in 2013 others 80 tadpoles were transferred from Porto Caleri to Bosco Nordio. An important milestone in the project occurred in 2014 when two spawn were found in a natural, not protected pond, one of which was hatched under protected conditions. The main objective of the project is to manage the *Pelobates fuscus* population in the wild in the best way so that it can stabilize in the future (Richard & Vianello, 2016).


(**Fig.9-10**; Adult individuals in Porto Caleri, the second one clearly shows the fossorial lifestyle of the animal, J.Richard)

One of the relevant objectives in the context of a conservation project, in addition to the demographic increase of the target species, is the preservation of genetic diversity, which represents the adaptive potential of the population itself, intended as the ability of a population to succeed in responding to selective pressures through molecular and consequently morphological changes. Some small populations have no possibility of migration and are forced to adapt in situ, so changing environments can cause selective pressures on individuals, altering their ability to successfully adapt. Selective pressures might have relevant effects on populations that suffer of low genetic diversity, for example an increased homozygosity caused by high levels

of inbreeding (Eizaguirre & Baltazar-Soares, 2014). The importance of preserving genetic variability is even higher in the case of species with low dispersal ability that can be due to intrinsic features of the species or to extreme habitat fragmentation in which suitable areas are separated by inhospitable environments that cannot be crossed. *Pelobates fuscus* has a low active dispersal ability and it is highly unlikely that the population of Bosco Nordio, as well as that of Porto Caleri, can be targeted my migratory flows that could increase their genetic diversity. It is then possible to hypothesize that the Bosco Nordio population, descending from a very small number of crosses, has a reduced genetic variability. This could represent a problem for the long-term survival of this population for two reasons: on the one hand, the reduced number of founders considerably increases the probability of inbreeding events, i.e. crossings between relatives with consequent high probability of homozygosity of potentially deleterious alleles and decreased chances of survival of individuals; the second potentially negative effect of reduced genetic diversity is the poor long-term adaptive potential of the population.

No information is available also for the population of Porto Caleri from which animals released in Bosco Nordio were taken. Its geographical location probably makes it poorly connected with other populations and it is possible that, due to a founding effect or prolonged isolation, it presents a reduced diversity. If confirmed, this could have important conservation implications: the genetic diversity of the Bosco Nordio population could be affected not only by being founded by a small number of individuals taken in Porto Caleri, but also by an already reduced diversity of the source population. It is therefore advisable to carry out, for the first time, a characterization of these populations, with the aim of providing suitable information for their long-term management. Additionally, it would be interesting to compare these populations with other ones of the same species, known to be demographically more consistent and possibly less isolated. In order to fill this gap of knowledge, samples for genetic analysis have been collected in the last 2 years both in Bosco Nordio and in Porto Caleri and the present study aims to lay the foundations for a continuous and long-term monitoring of these populations. Unfortunately, the available literature related to genetic analyzes on this species is limited to the analysis of mitochondrial DNA and in particular of CytB. Mitochondrial markers can be very useful in defining the phylogeographic relationships between populations but are scarcely informative for relatedness investigations, for which the use of biparental inheritance markers such as SNPs (Single Nucleotide Polymorphism) or microsatellites is more appropriate.

It is therefore a priority to make a preliminary effort for the isolation of polymorphic nuclear markers. In our case, the choice fell on microsatellites since SNPs, despite being extremely informative thanks to Genotyping by Sequencing techniques in which it is possible to simultaneously genotype hundreds of individuals at thousands of loci, require good quality DNA and above all in sufficient quantity for the construction of enriched genomic libraries. Since the samples currently

available are mainly oral swabs, which allow (and will allow in future years) minimally invasive sampling, it was considered appropriate to opt for microsatellites which can be characterized directly by PCR and do not require large quantities of DNA. The isolation and development of microsatellites for this species was therefore one of the main objectives of this thesis. The isolated markers were then applied both to the samples of Bosco Nordio and to those of the source population of Porto Caleri. For an external comparison, some samples from other populations were also analyzed.

2. THESIS PURPOSE

In extreme synthesis, within a collaboration with Veneto Agricoltura, the goal of this thesis work is to attempt the first genetic characterization of the *Pelobates fuscus* subpopulation of Bosco Nordio based on both nuclear and mitochondrial markers, and the comparison with the source relict population of Porto Caleri. In order to establish a long-term conservation program that aims at preserving these populations, in fact, it is a priority to evaluate the degree of existing genetic variability and to identify, if necessary, the best strategies for additional restocking interventions.

3. MATERIALS AND METHODS

3.1. SAMPLES DESCRIPTION AND EXTRACTION

Given that *Pelobates fuscus* species is extremely elusive, finding samples it is not straightforward; moreover, several populations have recently experienced dramatic declines all over Europe and therefore non-invasive sampling methods have been preferred to collect individual samples. Cotton swabs allow to collect buccal mucosal cells that can be used as a source of DNA for PCR-based analyses. In population studies, this non-destructive sampling approach seems to be particularly efficient for mitochondrial DNA sequencing and microsatellite typing. This method replaces the classic toe-clipping in amphibians, so it is surely less harmful for endangered species (Broquet et al., 2007). The collection of tissue samples was performed exclusively for already dead individuals.

In the present project, we used a total of 180 samples of *Pelobates fuscus* (Table 1) collected in different years from different research groups. In details, we used 65 DNA samples already extracted in 2019 in our laboratory. All of these samples were from labial and buccal swabs of metamorphosed toads. Specimens were collected

in two different localities in Veneto region: 51 from Porto Caleri and 14 from Bosco Nordio (Fig.6 in Introduction). In addition, I personally purified DNA from: 43 buccal swabs (20 of which derived from sibling metamorphosed toads) and 6 tissue samples all from Bosco Nordio collected in 2021, 6 embryos or eggs samples from Arsago Seprio (Lombardy), and 4 tissue samples from Cameri (Piedmont) provided by the 'LIFE *Insubricus*' project. In addition, we received 56 DNA samples, provided by Prof. Angelica Crottini (University of Porto), from different populations of *Pelobates*: 6 samples from Cameri (4 to be extracted), 1 from Arsago Seprio, 12 from Ivrea (Piedmont), 6 from Ukraine, 10 from Serbia, 10 from Germany and 11 from Croatia.

Origin	Lat	Long	Number of samples
Bosco Nordio	45.12501	12.26339	63
Porto Caleri	45.09648	12.33122	51
Arsago Seprio	45.68333	8.73333	7
Cameri	45.50121	8.66167	10
Ivrea	45.48128	7.90672	12
Ukraine	50.85000	33.26667	6
Serbia	44.83335	21.29998	10
Germany	52.26462	10.51096	10
Croatia	45.81444	15.97798	11

(**Table 1**. Number of samples and corresponding geographical origin. Latitude and longitude are expressed in decimal degrees.)

Total DNA was purified with the DNeasy Blood & Tissue Kit of Qiagen® following the protocol here below:

- Tissues or swabs were treated with 20 µl Proteinase K and 180 µl Buffer ATL at 56°C for 30/45 in a thermomixer (ThermoMixer F2.0, Eppendorf®) for the complete lysis of the tissues or the detachment of cells from the swabs.
- After addition of 200 µl Buffer AL (with added ethanol) and incubation at 56°C for 10 min, the lysate mixture was pipet into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at ≥6000 x g for 1 min to allow DNA binding to the silica-based membranes.
- Impurities were removed by thorough washing with Wash Buffers (500 µl Buffer AW1 followed by centrifuge for 1 min at ≥6000 x g and 500 µl Buffer AW2 followed by centrifuge for 3 min at 20,000 x g to dry the DNeasy membrane).

- The genomic DNAs are then eluted in low salt Elution Buffer by pipetting 200 µl Buffer AE directly onto the DNeasy membrane and centrifuging for 1 min at ≥ 6000 x g.

For every centrifugation the Centrifuge 5420 Eppendorf® with a 24 spin columns capacity was used. Given the low concentration found in the DNA extracted from buccal swabs, the elution step was performed in triplicate trying to recover the major part of the DNA from the membrane.

The genomic DNAs were quantified using the spectrophotometer NanoDrop 2000c (Thermo Scientific™). This instrument can measure the absorbance of the genomic DNA at different wave lengths. Using 1,5 µl of extracted DNA, the measurement of the absorbance at 260 nm allows to estimate the concentration of the nucleic acid in ng/µl. In addition, it is also possible to evaluate the purity and the presence of contaminants in the samples checking the 260/280 and 260/230 absorbance ratios (García-Alegría et al., 2020).

To evaluate the integrity of the DNA molecules, extracts were also visualized on 1% agarose gel in TBE 1X stained GelRed (Biotium; GelRed™ Nucleic Acid Stain 10,000 in water).

3.2. MITOCHONDRIAL MARKER: CYTB

3.2.1 CYTB AMPLIFICATION, PURIFICATION AND SEQUENCING

Mitochondrial cytochrome b gene is one of the most widely used molecular markers in phylogenetic studies due to the in-depth knowledge about the structure and function of the protein encoded by the gene (Esposti, 1993). Moreover, its utility has already been tested numerously in different taxonomic entities of vertebrates to estimate population-level relationships (Farias et al., 2001). For these reasons, in order to observe divergence between different groups, cytochrome b sequences were analysed from samples of *Pelobates fuscus*.

Primers L15162 (5′GCAAGCTTCTACCATGAGGACAAATATC-3′) and H15915 (5′-AACTGCAGTCATCTCCGGTTTACAAGAC-3′) (Crottini et al., 2007) were used for the amplification of 702 bp of the mitochondrial cytochrome b gene.

The amplification was performed in a final volume of 20 µl containing Buffer 1X (Wonder Taq reaction Buffer Euroclone), primers 1 µM each and Taq 1 U (Wonder Taq DNA Plymerase Euroclone). For each PCR, about 10 ng/µl (when possible) of genomic DNA were used. The PCR profile consisted of denaturation for 1 min at

95°C, followed by 35 cycles of 95°C for 15 s, 50°C for 15 s and 72°C for 45 s, and a final single step at 72°C for 2 min.

The results of the amplifications were visualized on agarose Gel 1.8% in TBE1X stained with GelRed.

For some individuals with low concentrated DNA, increased amount of DNA templates was processed in the attempt to obtain PCR products of good quality for sequencing. All PCR products successfully obtained were purified with the EuroSAP PCR Enzymatic Clean-up kit (EuroClone) following the manufacture protocol. This kit takes advantage of two enzymes, the Exonuclease 1 (Exo1) that degrades single-stranded DNA primers and the Shrimp Alkaline Phosphatase (SAP) that has the role to dephosphorylate unused nucleotides. The reaction was performed in a total volume of 7 µl including 5 µl of PCR product and 1 µl of each enzyme and incubated at 37°C for 5 min. Finally, heat inactivation was performed at 80°C for 10 min.

All sequencing reaction was performed by Eurofins Genomics external service (www.eurofinsgenomics.eu).

3.2.2. MITOCHONDRIAL DATA ANALYSIS

Chromatograms of sequencing were visualized, manually checked and analysed by MEGA 11 (Tamura et al., 2021). Sequences were aligned with Clustal W.

For the analysis, three datasets were created. The first included all sequences 745 bp long obtained in this study from Bosco Nordio and Porto Caleri. For the second dataset, we also included sequences from our samples of Arsago Seprio and Cameri and the 51 out of the 52 cyt-b sequences of the samples received by Dr.ssa Crottini for comparison. A third dataset was composed by adding to the previous set of sequences haplotypes W1-W28 and E1-11 from Crottini et al. (2007) work.

Haplotype genealogies with both datasets were constructed with the TCS method (Clement et al., 2000) implemented in PopArt software (Leigh & Bryant, 2015).

3.3. NUCLEAR MARKERS: MICROSATELLITES

3.3.1. MICROSATELLITE ISOLATION AND PRIMER CONSTRUCTION

All microsatellite loci used in the present study were obtained ex novo, by the external service GenoScreen (https://www.genoscreen.fr/en/), through an Illumina

MiSeq Nano 2x250 v2 sequencing of enriched libraries from 5 DNA of adult individuals of *Pelobates fuscus* sampled in Bosco Nordio.

The obtained raw sequences (2.059.256) were merged with Usearch software (Edgar, 2010) and screened for microsatellite detection and primer construction with QDD v3 software (Meglécz et al., 2010), producing 54.583 merged sequences and primer pairs for 207 loci. Given that the library yield is low for this species, GenoScreen performed a second more flexible merging with Prinseq software (https://prinseq.sourceforge.net/). This resulted in a total of 1.166.562 merged sequences and 2560 primer pairs (which include the previous best selection of loci).

Focusing on the best selection of 207 microsatellite loci, we selected a total of 41 loci (23 at first then increased with other 18) for the genotyping. Loci with perfect microsatellite with continuous repeat of single motif, high number of repeats, and amplifiable size of the PCR product (compatible with multiplexing) were preferred when possible.

3.3.2. MULTIPLEX-PCR REACTIONS

The selected primer pairs were analysed using FastPCR software (Kalendar et al., 2017) to simulate in silico multiplex PCR, check cross-dimers formation and set up the best multiplex composition. After a first primer test in single-locus PCR to verify the successful amplification of selected loci by using our DNA samples, forward primers of 37 out of 41 loci were labelled with fluorescent dyes (FAM, HEX, TAMRA, ATTO565) for detection with ABI PRISM automatic sequencer and amplified together in four separate multiplex reactions (Table 2).

Multiplex-PCR is a method that allows the simultaneous amplification of different loci by using locus-specific primers into the same reaction mixture (Markoulatos et al., 2002).

A total of four multiplex reactions were set up, MA, MB, MC, and MD, including 10, 9, 9 and 9 loci respectively (Table 2). All reactions were performed in a final volume of 10 µl including 1X MasterMix (QIAGEN, HotStarTaq DNA Polymerase, Multiplex PCR Buffer, dNTP Mix), 0.2 µM of each primer and 20 ng/µl DNA template. The thermal profile was as follows: initial activation step for 15 min at 95 °C, 35 cycles of 30 s at 94 °C, 90 s at 60 °C and 1 min at 72 °C, and final elongation for 30 min at 60 °C.

3 loci of the MA, 4 of the MB, 2 of the MC and 1 of the MD were amplified individually with a different annealing temperature detailed in Table 2 and included within the correspondent multiplex reaction for genotyping. Loci with unclear genotypes were also re-amplified and genotyped individually.

Locus	Primer sequences (5' -> 3')	Ta (°C)	Size (bp)	Motif	Multiplex
1509	F-CCCTGTTTAGCGAGTATTATCAGG (ATTO565) R-ATCACAACTCTCCCAGAGGTT	60	190	$(AC)_9$	MA
19528	F-ACACAGGCGAAGATGCATACA R-TGTATGCCTGGATGTCTCTGT		139	$(ACAT)_7$	
1167*	F-TTCCAGTCCCATCTTGTCCC (ATTO565) R-ACGAAGAGCCACATTTCCGT	60	128	$(AG)_7$	MB
11806	F-ACAGCTAGAGCATGCAGAGC (FAM) R-ATGTAGAAAGGTACTGCTGGG	60	290	$(AT)_6$	MB
14420	F-GGATCCTCCTAGGGTTGTATTCT (HEX) R-TTTCACCCTGTAGCTGTCCT	60	233	$(AC)_{10}$	MB
16554*	F-ACCTGTTGTCTCACATGTCCC (FAM) R-ACCGAAACCATTGATGAGTGT	60	169	$(AG)_{10}$	MA
19412*	F-ACAGGCACAAGTAGCTCACC (HEX) R-GTGGGCTGGTAGACACTTCC	63	216	$(ATC)_9$	MA
19497*	F-TGGAGGATCATGACCAGCTG (TAMRA) R-TCCTTGAATTCACGACAATTCTCAC	61	240	$(AC)_8$	MB
3736*	F-GCTTTCTGACACTCTGTTCAGG (HEX) R-CCGGTCCTGTTGACGACTAC	60	144	$(AT)_8$	MB
25215	F-AAGGTTATGAGAGGGTCAACAAA (HEX) R-TTTGAAACAGCCGGTCGGAA	60	290	$(AC)_6$	MA
25207	F-ATTGTACGTGTCAGCCAGGG R-AGTCCACCCATTGTAGAGCG		154	$(AAT)_6$	
18490*	F-GGAGACACCCACTAGGACCT (TAMRA) R-TTGCTGGCATACGGTTTGTG	58	299	$(AG)_5$	MB
12801	F-AGTGATCTGCCCTAGCCTGA (TAMRA) R-TGAAGATAATTCCTGACCTCCCA	60	122	$(AT)_7$	MA
20472*	ATGCTTCTGGGCACAATGGA (FAM) CACCCTCCCATTACCTGAGC	61	253	$(AT)_8$	MA
17013	F-GGGTCTGGAGTGTCCCTTTAA R- TCAAGCAAGCCTCTGACAGG		178	$(AC)_8$	
18843	F-TGGTGTCTTCTCCAGCACAG (ATTO565) R-TCAGGAACGGAACCCATGTT	60	259	$(AT)_7$	MB
17465	F-AGTAGGTGTAGCAGGTGGGT (HEX) R-ACCACCGCGATGATCAAGTT	60	144	$(AG)_6$	MA
17493	F-AGCTGTAGTTGTTCTGTTGACG (FAM) R-CCATGGCAGCTTGAGGGTTA	60	117	$(AC)_6$	MA
22699	F-CACCACTTTGTACCCATCGGA (TAMRA) R-GCTTCTAAGGAGTGGATGGGC	60	194	$(AT)_8$	MA
10041	F-ACTTTAGCAAGGAAATTTGGCAGA (TAMRA) R-AGGTATAGCGCAATCGGGTG	60	276	$(AC)_6$	MA
6454	F- ACCCATTCTTGGCAGATCCT R- GCCACTTGAGGTGTCCCTAG		228	$(AT)_6$	
7132	F-CCAGCGTTTAGGACCAGGTC (FAM) R-CCTTCTCATTGTGCAGGCAC	60	197	$(AT)_6$	MB
14091	F-AATGCATTAAAGGCAATGATAACCA (HEX) R-AGCAAATGAATGTAGCCCTTTGC	60	290	$(AG)_5$	MB

13975	F-TGTAATGTTTGCGTTTGCCGT (FAM) R-CAAACATGCTTACATTCAAACACA	60	131	$(AC)_8$	MC
10892	F-AAGTCCATGCCTCGCTGTTT (FAM) R-CGTGTACGCTATGGAATAAACCA	60	199	$(AC)_6$	MC
22119*	F-TCATGGAGAAGCCTCTGTGC (FAM) R-TCCATGTCTGAAGTCTGTCACT	58	292	$(AC)_5$	MC
19753	F-GCACCTGCATACACAACACA (HEX) R-TGTCTGCAAGCCAGTTAATGAC	60	152	$(AT)_6$	MC
8594	F-AAGCTGTCACCCACATAGCAA (HEX) R-ACTTAGTAAAGTGGTAATTATGGCACA	60	241	$(AC)_6$	MC
15035*	F-TCTCAAATAGAGCTTCTCCAGTCAG (HEX) R-GTGGCGATCACGTGGTTAC	62	290	$(AC)_5$	MC
8689	F-CCTCGTCAAGTGTTATAGAAAGTTAT (TAMRA) R-GGAGTTTAACATGCGCCTTCC	60	167	$(AG)_6$	MC
12396	F-CCCATGCTTTGCAGATGTGTT (TAMRA) R-CCTCAATATTCACAGCCTGACA	60	239	$(AC)_6$	MC
12865	F-ACCAAATCTTACCACCTGCTTT (ATTO565) R-GGGACATTAGGATAGAGAAGAGGG	60	195	$(AG)_6$	MC
6602	F-TGGACTGGCAGATGAGGTTT (FAM) R-TAGGCGAATCTGGAGTCCCT	60	138	$(AAT)_7$	MD
4942	F-GGCAAGGCAGCAAATAGATTGT (FAM) R-TGTCTACTGCTAGACTGTTAGAACT	60	197	$(AC)_6$	MD
12217*	F-CTCCATTGAACACATGGCAGT (FAM) R-GCTCTGGCGTTCGGTAGATT	62	257	$(AC)_6$	MD
8042	F-GGAAGCAGATCAAGCAGCCT (HEX) R-ACACATAACCTTCCTCACTCCC	60	162	$(AC)_6$	MD
15210	F-GGGTTTATTGCAGCACCTGT (HEX) R-GTTGTGCTCTGGCTGCCTAT	60	252	$(AG)_9$	MD
2998	F-GAGCAGAGCCTGTTCTAGCT (HEX) R-GCCATTGTGGTCACTGCAAA	60	299	$(AAAC)_5$	MD
3053	F-GACACCTGTCATACTACAGCA (TAMRA) R-TCCCATTGTACAGCGCTACG	60	169	$(AAT)_6$	MD
10102	F-CATTTCCTGGTCTGCCCTGT (TAMRA) R-ACAACTATACCCAGCTGTAGGC	60	221	$(AGAT)_9$	MD
6587	F-CTGTGCGTCCAACTCGTCTT (ATTO565) R-GTAGCGTACCGGAGCGAG	60	194	$(AAT)_6$	MD

(**Table 2**: Details about all microsatellite loci analysed in the present study. For each locus, the name, primer forward and reverse, the fluorescent dye, the annealing temperature (Ta), the expected amplicon size, the repeat motif with the number of repeats and the multiplex they belong to are reported. Loci excluded for inefficient pairing of primers are highlighted in red. Asterisks (*) indicate loci amplified in single PCR reaction and then assembled in the corresponding Multiplex.)

Amplifications were always checked on 1.8% agarose gel in 1X TBE buffer with GelRed (Biotium).

Fragment genotyping was performed by an external service, BMR Genomics in Padua (http://www.bmr-genomics.it), on an ABI 3100 automated sequencer

(Applied Biosystems). Genotyping consists in capillary electrophoresis (CE), inside a small-diameter quartz capillary, of fluorophore-labelled DNA fragments for their size-based separation (Smith & Nelson, 2004). To ensure reproducibility of results and minimize differences due to capillary electrophoresis, about 10% of all specimens were re-genotyped and re-scored.

Scoring was performed with GeneMarker® software v1.95 (SoftGenetics). The process consists of identifying the peaks in a chromatogram corresponding to the alleles, for each microsatellite locus (Flores-Rentería & Krohn, 2013).

The final dataset included 139 individuals genotyped at 12 loci formatted as Genepop input file (Rousset, 2008). Unfortunately, the amount and concentration of some DNAs did not allow the amplification of all loci. In these cases, we decided to give precedence to more polymorphic loci for genotyping and this why the final dataset obtained was partially incomplete and microsatellite analysis were performed considering a subsample of loci.

3.3.3 MICROSATELLITE DATA ANALYSIS: GENETIC DIFFERENTIATION

Microsatellite analysis were performed considering a total of 139 individuals from 8 populations genotyped at 12 polymorphic microsatellite loci (16554, 20472, 19412, 25215, 22699, 1509, 7132, 11806, 14420, 19487, 18490, 18843). The remnant individuals or loci were discarded due to the presence of too much missing data.

The conversion of the input files for the analysis was performed with CREATE (Coombs et al., 2008) or Genepop on the web (https://genepop.curtin.edu.au/).

The HP-Rare software (Kalinowski, 2004) was used to perform descriptive statistical analysis as allelic richness (Ar) and the number of alleles per sample, compensating for the disparity in numerosity (considering as the lowest samples size 7 individuals). Through the rarefaction technique, the algorithm produces an equivalent measurement in different sample sizes.

Expected and observed heterozygosity per locus and population were measured with Arlequin ver 3.5 (Excoffier & Lisher, 2010) software. Allelic frequencies were acquired by Genepop version 4.7.5 (Rousset, 2020) population genetics software. This value represents the fraction that each detected allele occupies within the locus.

3.3.4. MICROSATELLITE DATA ANALYSIS: POPULATION DIFFERENTIATION

Population differentiation was investigated using ARLEQUIN 3.5 (Excoffier & Lischer, 2010) by non-hierarchical and hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992) and pairwise FST estimation following Weir & Cockerham (1984). FST values represent the deviation from panmixia by individuals (Wright, 1943) and are calculated as a ratio of variance components that are obtained from a matrix of squared Euclidean distances between pairs of individuals (Excoffier et al., 1992).

Significance of differentiation was determined using 10000 random permutations of individuals among populations and, when needed, populations among groups. The Benjamini-Hochberg correction (Benjamini & Hochberg, 1995) was applied in case of multiple tests.

Patterns of population structure were investigated through a clustering analysis with Structure v2.3.4 (Pritchard et al., 2000). The software is a useful tool to assess a genetic classification in potential populations, through evaluation of allele frequencies at a series of unlinked loci. Structure uses a systematic Bayesian clustering approach applying Markov Chain Monte Carlo (MCMC) simulation and tests different, assumed genetic groups (K) (Porras-Hurtado et al., 2013). For our dataset, a burn-in period of 100.000 and a running length of 1.000.000 of MCMC were applied to test for K from 1 to 10 (10 replicates each K). A second run with Italian population only was performed testing K from 1 to 7 (10 replicates each K).

The best number of K was calculated using StructureHarvester program (Earl & vonHoldt, 2011). Through the program it is possible to observe the mean likelihoods per K value. In addition, the algorithm of the software implements the execution of the ‘‘Evanno’’ method, that uses ΔK as a good predictor of the real number of clusters (Evanno et al., 2005).

Structure multiple runs for each K value were post-processed using Clumpak (Kopelman et al., 2015). Clumpak takes advantage of a Markov clustering algorithm to identify similar runs and divides them in separate groups among all possible solutions, obtaining a consensus.

Principal component analysis (PCA) was carried out with R software (R core team, 2017). PCA aims to reduce the dimensionality of the data while retaining most of the variation in the totality of the data. This is achieved by determining axes, representing principal components, along which the variation in the data is greatest. The result can be represented on a plot, which allows visualization of distances and formation of clusters (Ringnér, 2008). In R, “ggplot” function was used to create

the graphs. A Genepop file with 116 individuals for 12 microsatellite loci was used to graphically represent Italian populations (Bosco Nordio, Porto Caleri, Arsago Seprio, Cameri and Ivrea). For Italian and European populations (Croatia, Serbia and Germany), the dataset was composed by 139 individuals for 12 loci.

4. RESULTS AND DISCUSSION

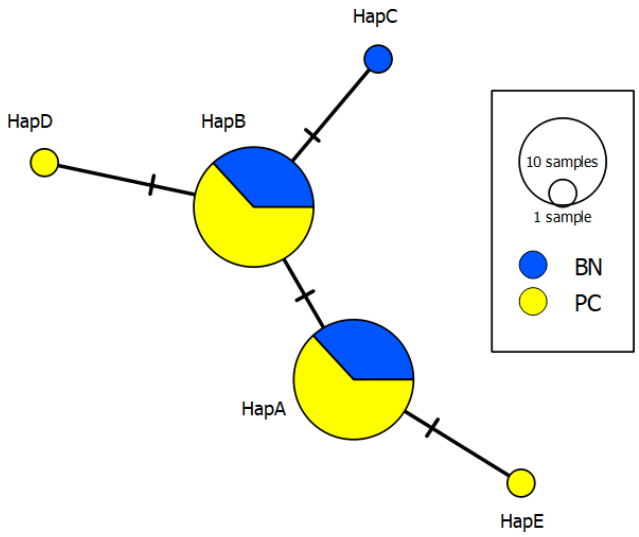
Out of a total of 124 samples on which DNA purification was carried out, 49 yielded DNA of a quality and concentration such suitable to perform mitochondrial DNA analyses and 104 for microsatellite analyses.

In some cases, poor quality or quantity DNA was obtained, mostly from samples collected using buccal swabs. This approach, besides having an intrinsic variability due to the differences between animals (size, reactivity, hydration state, etc.) provides samples that must be preserved by freezing, which must be timely to avoid rapid degradation of the collected cells. A rapid freezing is not always possible during in-field sample collection. This sampling approach, however, has only recently been tested at the Bosco Nordio reserve and requires standardization of the protocol as well as operator training.

In addition to these, samples received from Dr. Crottini as described in Materials and Methods were used for analyses.

4.1. MITOCHONDRIAL DATA ANALYSIS

Analysing a region of 745 bp, a total of 5 cytochrome b haplotypes were detected in the two populations of Bosco Nordio (BN) and Porto Caleri (PC). A network among these haplotypes, built using the software TCS method implemented in PopART (Leigh & Bryant, 2015), using all 41 sequences, is reported in Figure 11.

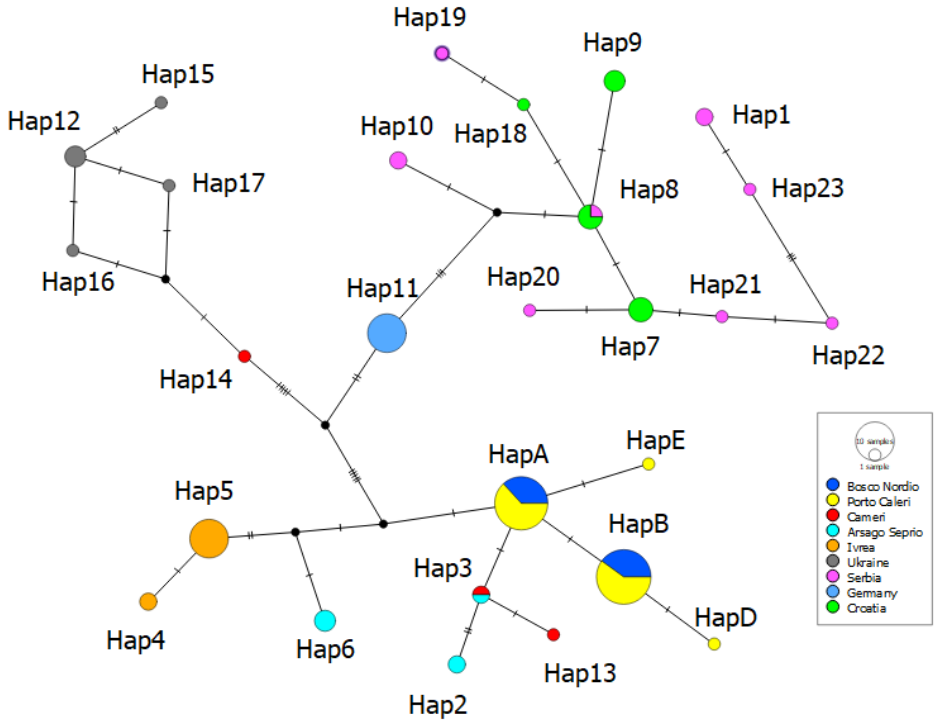


(**Fig.11**: TCS network for Bosco Nordio (BN) and Porto Caleri (PC) populations in which each circle represents a different haplotype. Size of circles is proportional to the number of specimens that bear a given haplotype. Black bars represent single nucleotide substitutions.)

Haplotypes A and B, which differ by one nucleotide substitution, resulted to be the more frequent both in PC (12 and 12 respectively) and in BN (7 and 7 respectively). Additional three haplotypes were observed all separated from Haplotypes A or B by a single mutational step difference.

Several hypotheses can be advanced to explain the genetic homogeneity of the haplotypes observed in PC (and consequently in BN). The first is that this homogeneity is characteristic of the populations of the eastern Po Valley and the second is that the population of PC, being ecologically isolated from inhospitable environments, was founded by a very small number of individuals and that, following the isolation, the observed diversity was generated on site. To evaluate these hypotheses, it would be interesting to analyse individuals from other, possibly less isolated oriental populations, in order to evaluate if the variability in PC is only a part of a higher oriental variability.

In order to be able to insert the haplotypes observed in a broader picture of diversity, a comparison was made with the samples provided by the LIFE *Insubricus* project (Arsago Seprio and Cameri) and genotyped during this study and with the 51 out of the 52 sequences provided by Dr. Crottini from different geographical locations. For coherence with the latter sequences, the considered region was reduced from 745 to 702 bp which led to the exclusion of the polymorphism that gave rise to the C haplotype (Fig.11) and its collapse within the B haplotype. Figure 12 shows the haplotype network of the analysis described above.



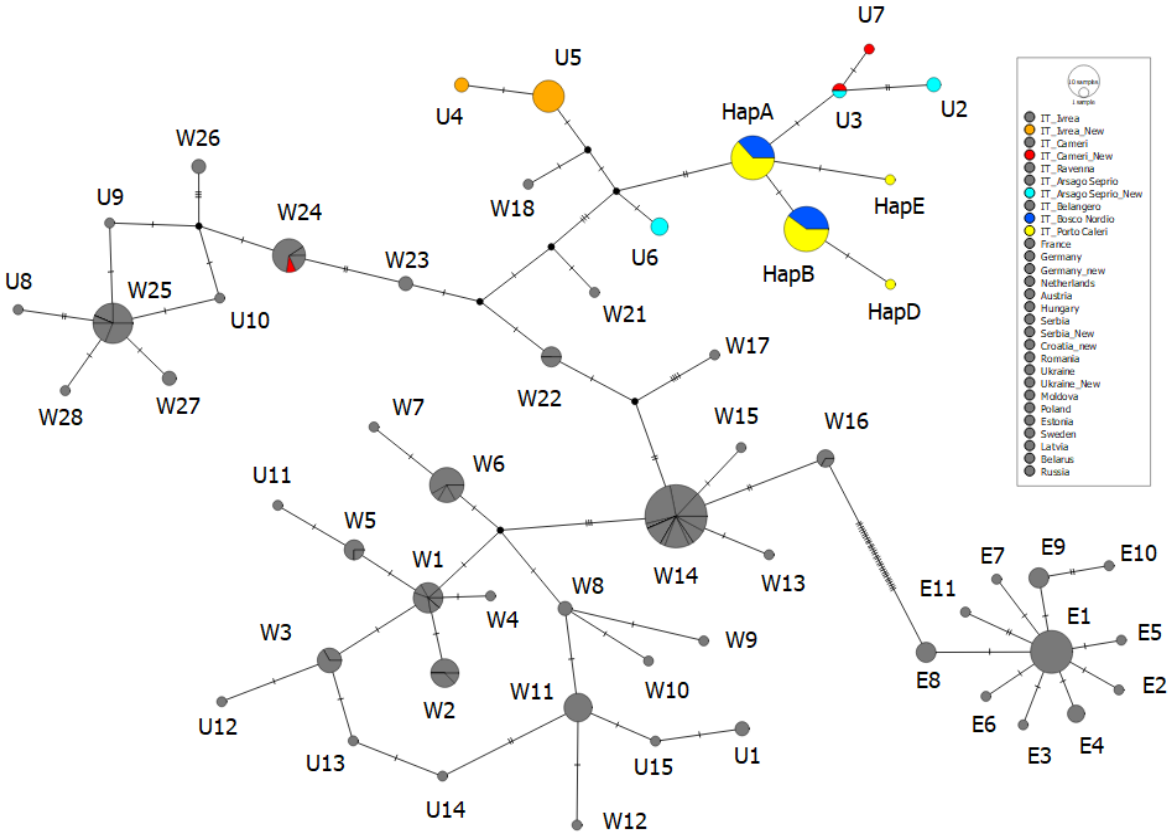
(**Fig12**: Haplotype network of 702 bp cytochrome b sequences, including PC and BN populations as well as Cameri, Arsago Seprio, Ivrea, Ukraine, Serbia, Germany and Croatia. Haplotypes were named by assigning them a number from 1 to 23 (Hap1 – Hap23) and colours represent a different geographic area according to the legend.)

The network reported in figure 12 shows how the Italian populations group together and are separated from those of other areas. This result was expected on the basis of published data (Crottini et al., 2007) but is confirmed following the addition of the sequences generated by us. As far as the oriental populations are concerned, the haplotypes of PC and BN, although falling within the cluster of Italian sequences, are not shared with other populations.

Locality	Sample size	Haplotypes
Bosco Nordio	15	7 x HapA; 8 x HapB
Porto Caleri	26	12 x HapA; 12 x HapB; HapD; HapE
Cameri	3	Hap3; Hap13; Hap14
Arsago Seprio	6	2 x Hap2; Hap3; 3 x Hap6
Ivrea	12	2 x Hap4; 10 x Hap5
Ukraine	6	3 x Hap12; Hap15; Hap16; Hap17
Serbia	10	2 x Hap1; Hap8; 2 x Hap10; Hap 19; Hap20; Hap21; Hap 22; Hap23
Germany	10	Hap11
Croatia	11	4 x Hap7; 3 x Hap8; 3 x Hap9; Hap18

(**Table 3**: Number of sequences used for network construction and number and type of haplotypes identified for each geographic area.)

To further extend the comparison, haplotypes published in the work of Crottini et al. (2007) downloaded from GenBank were also included in an additional network created again considering the 702 bp length of the cytb sequence. These additional sequences were obtained from 156 *Pelobates fuscus* individuals sampled from 61 different localities distributed in almost the entire range of occurrence of the species in Eurasia; in particular, 28 haplotypes of the "western" type (W1 – W28) and 11 haplotypes of the "eastern" type (E1 – E11) were identified.



(**Fig.13**: Haplotype network in which the haplotypes identified in the work of Crottini et al. (2007), downloaded by Genbank, were added to the sequences used in the previous network. Some of the haplotypes are shared with the network in Fig 12 but the names were changed according to the ones published. For the haplotypes of BN and PC we left the original name. The prefix "IT" indicates Italian provenance; the suffix "New" indicates samples not included in the work of Crottini et al. (2007). Extra-Italian and published haplotypes are in grey. Correspondence between haplotype names in the two networks is reported in Table 4).

The observed pattern does not change significantly compared to that of figure 12. It is interesting to observe the high degree of differentiation of the eastern lineage (Crottini et al., 2007) more recently considered as a separate subspecies (Litvinchuk et al., 2013).

Focusing on the populations of the eastern Po Valley covered by this study, we can conclude that, on the basis of mtDNA, the two populations have a limited variability but not dissimilar from that of other Italian populations. The fact that all the oriental haplotypes have never been observed in other populations suggests a certain degree of isolation which, however, should be verified on the basis of a more representative sampling of the populations of this area.

new name	corresponding	new name	corresponding
U1	Hap1	U7	Hap13
U2	Hap2	W24	Hap14
U3	Hap3	U8	Hap15
U4	Hap4	U9	Hap16
U5	Hap5	U10	Hap17
U6	Hap6	W5	Hap18
W3	Hap7	U11	Hap19
W1	Hap8	U12	Hap20
W2	Hap9	U13	Hap21
W6	Hap10	U14	Hap22
W14	Hap11	U15	Hap23
W25	Hap12		

(**Table 4**: Correspondence between haplotypes name of Fig.13 and Fig.12 networks).

This analysis allowed the identification of a group formed by the Venetian populations, genetically isolated from the rest of Italy and even more so from Europe. The age of the population of Porto Caleri is not known, but it was ruled out as being of recent origin (Boschetti et al., 2006).

A study by Eggert et al. (2006) states that being amphibian very sensitive to changing environmental conditions, current population genetic structure can arise from paleoenvironmental fluctuations. The study shows a low intraspecific haplotype difference by cyt-b sequencing, suggesting recent expansion from a single source area. In addition, the current geographical distribution is characterized by a genetic structure determined by low gene flow and distance-dependent isolation.

The work of Crottini et al. (2007) based on cyt-b analysis, suggested the existence of the Po Valley as a glacial refuge for *Pelobates fuscus* species in the Pliocene. Refugia have the role of biodiversity hot-spot with a high degree of genetic variability. As a result of climate change, spread to other environments would have occurred, resulting in high population structuring due to repeated founder effects associated to reproductive isolation.

What can be hypothesized is that, due to the proximity of the Porto Caleri locality to the mouth of the Adige River, individuals of spadefoot toad were able to colonize this geographic area using the waterway as a corridor or, more likely, trough adult migration on emerged lands. As already described in the introduction, the area presents a suitable environment for the survival and reproduction of this particular amphibian, so a spreading phenomenon from a nearby geographic area cannot be ruled out. Regarding the origin of the population of *Pelobates fuscus* from Porto Caleri, some hypotheses can be made, which at the present time cannot be verified, considering also the absence of historical data for the southern part of the province of Venice and for Polesine.

This population (as well as that of Porto Fossone) is located in a relatively recent area of the plain, originated by the deposition of sediment from the Po River (which until 1700 flowed halfway along the shoreline of the Rosolina-Porto Caleri peninsula, with its northern branch, the Po di Tramontana) and the Adige River.

The dune cordon that originated about a millennium of years ago, still recognizable today in some relicts (e.g., Bosco Nordio, the fossil dunes of Taglio di Po and Volto di Rosolina) presented, toward the coast, a lagoon and marshy belt of brackish water, of which the Caleri Lagoon remains as evidence. These environments presumably were not very hospitable to the presence of a freshwater-bound amphibian with low saltwater tolerance (Stanescu et al., 2013) such as the *Pelobates fuscus.*

The only favourable habitats were probably found either upstream of the dune cordon mentioned above, where there were large expanses of freshwater marshes, or along the course of the Adige and Po rivers.

In particular, then, the Rosolina-Porto Caleri peninsula, which was already very similar to the present conformation in the maps of the late 1700s, had, at least until the first half of the 20th century, very different habitats from the present ones, since, as can also be seen in the aerial photos of the 1950s, the coastline consisted of dune habitats without tree cover and without any appreciable size of wetland habitats (Fig.14).

Thus, as mentioned, the only freshwater inputs from the rivers, may have produced suitable habitats for the reproduction of the species along the river shaft, and acted as pathways for the colonization of these environments.

The colonization of the habitats now used could therefore be speculated to have occurred recently (second half of the 20th century, at least for Porto Caleri) from two different points: either from dulcicultural habitats preserved in the Caleri Lagoon (in the fishing valleys present there), along the edge of the dune cordon of the peninsula or (more likely), from the Adige River where, in the locality of Porto Fossone, a large wetland now regimented by a water-scooping machine had long been present. Thus the colonization of the locality of Porto Caleri would result from a later colonization, which would have seen the arrival of animals from inland parts of the plain along the course of the Adige, the stabilization of populations near its course and the locality of Porto Fossone, and the subsequent movement of animals to the end of the peninsula (Porto Caleri) perhaps using ephemeral breeding habitats located along the peninsula itself.

(**Fig.14**: The area of the mouth of the Adige River, with the peninsula that currently runs from Porto Fossone ("Sbocco di Adige") to Porto Caleri. About halfway down the then already abandoned mouth of the Po di Tramontana.)

4.2. MICROSATELLITE ANALYSIS

4.2.1 MICROSATELLITE LOCI ISOLATION

23 loci were initially selected from the selection of 207 best microsatellites made by the Genoscreen service (https://www.genoscreen.fr/en/). A primer test in single-locus PCR to verify the successful amplification of selected loci with our DNA samples was done. 4 out of the 23 loci were discarded due to the absence of a clear

PCR product (Table 2, Materials and Methods). The other loci that were amplified in Multiplex, and subsequently genotyped, showed a low degree of variability. Only a few were polymorphic for the Italian samples analysed, while they showed higher diversity in European populations.

Looking for loci with higher variability, 18 additional microsatellites were selected for primer designing, testing and assembling into two other Multiplexes (MC-MD). After preliminary genotyping of 4 individuals from different Italian populations, these microsatellites also showed a low degree of variability. For this reason and because of the scarcity of remaining DNA, they were used only for samples from non-Italian populations that showed a higher degree of polymorphism.

4.2.2 GENETIC DIFFERENTIATION

4.2.2.1. ALLELIC RICHNESS (Ar)

	AVG OVER LOCI	16554	20472	19412	25215	22699	1509	7132	11806	14420	19487	18490	18843
Bosco Nordio	1.57	1.9999	1.9865	2.9527	1.9459	1.9998	1	1	2	1	1	1	1
Porto Caleri	1.65	1.9282	2.745	2.9999	2.1733	1.9986	1	1	2	1	1	1	1
Arsago Seprio	1.83	2	2	4	1	2	3	1	2	2	1	1	1
Cameri	1.5	1	1	4	2	1	1	1	3	1	1	1	1
Ivrea	1.46	1.9904	1	2	1	1	2	1	2.9904	1.5833	1	1	1
Croatia	2.78	3.8667	2	7.8441	1	1	4.8775	2	1.7778	2.9559	1.9951	1	3
Germany	2.25	2	1	4	2	1	3	3	1	4	3	2	1
Serbia	2.92	5	2	7	1	2	3	2	3	2	3	1	4

(**Table 5**: Allelic richness (Ar) measured in 129 individuals divided in 8 populations, for each of 12 loci considered, through HP-Rare software (Kalinowski, 2004). Only 1 out of the 11 genotyped siblings from BN was considered.)

Values of allelic richness, the diversity represented by the average number of alleles per locus (Leberg, 2002), are reported in Table 5. The average number of alleles per locus among the Italian populations ranges between 1.46 and 1.83, lower than the average of the European populations considered, ranging between 2.25 and 2.92. The population of Ivrea shows the lowest value of allelic richness while the highest is found in Serbia.

The population in BN has slight lower allelic richness than the source population in PC, result that may suggest a bottleneck imposed by the translocation of a limited number of animals.

4.2.2.2. HETEROZIGOSITY

Microsatellite loci were used as molecular markers to estimate the degree of heterozygosity in both Italian and European populations of *P. fuscus*.

		Bosco Nordio		Porto Caleri		Arsago Seprio		Cameri		Ivrea		Croatia		Germany		Serbia	
		Obs Het	Exp Het	Obs Het	Exp Het	Obs Het	Exp Het	Obs Het	Exp Het	Obs Het	Exp Het	Obs Het	Exp Het	Obs Het	Exp Het	Obs Het	Exp Het
1	**16554**	0.56667	0.49435	0.28000	0.27152	0.14286	0.14286			0.36364	0.31169	0.50000	0.69167	100,000	100,000	0.85714	0.72527
2	**20472**	0.32143	0.36299	0.48980	0.47865	0.28571	0.26374					0.33333	0.33333			0.50000	0.50000
3	**19412**	0.62069	0.58076	0.62500	0.55636	0.71429	0.65934	0.57143	0.73626	0.83333	0.50725	0.66667	0.89542	0.71429	0.57143	100,000	0.87912
4	**25215**	0.26667	0.28249	0.22000	0.21980			0.14286	0.14286					0.14286	0.14286		
5	**22699**	0.66667	0.48814	0.42000	0.45960	0.28571	0.26374									0.00000	0.42857
6	**1509**					0.14286	0.38462			0.00000	0.50725	0.44444	0.72549	0.42857	0.56044	0.16667	0.59091
7	**7132**											0.33333	0.50327	0.28571	0.58242	0.00000	0.52747
8	**11806**	100,000	0.50847	100,000	0.50505	100,000	0.53846	100,000	0.67033	100,000	0.65801	0.11111	0.11111			0.28571	0.61538
9	**14420**					0.71429	0.49451			0.08333	0.08333	0.33333	0.46405	0.42857	0.64835	0.42857	0.49451
10	**19487**											0.33333	0.29412	0.60000	0.51111	0.57143	0.71429
11	**18490**													0.00000	0.53333		
12	**18843**											0.00000	0.71429			0.42857	0.49451

(**Table 6**: Observed heterozygosity and expected heterozygosity compared, for each of the 12 loci in the 129 total samples, divided in populations. Homozygotes loci are represented by a grey cell. Only 1 out of the 11 genotyped siblings from BN was considered.)

There is no marked difference in heterozygosity between different populations, apart from a few more variable loci. The most important result is the number of heterozygous loci that are present in the populations. In fact, extra-Italian populations have a greater number of polymorphic loci.

There is no particular divergence in heterozygosity also within *P. fuscus* Italian populations. Focusing on individuals from BN and PC, this degree of similarity might suggest that the source population also exhibits some degree of inbreeding. Unfortunately, there is not enough information to state this, as there is not much information about the remaining populations in Italy.

4.2.2.3. ALLELIC FREQUENCIES

Allelic frequencies of the 12 loci were measured for 129 genotyped individuals, thanks to Genepop version 4.7.5 (Rousset, 2020).

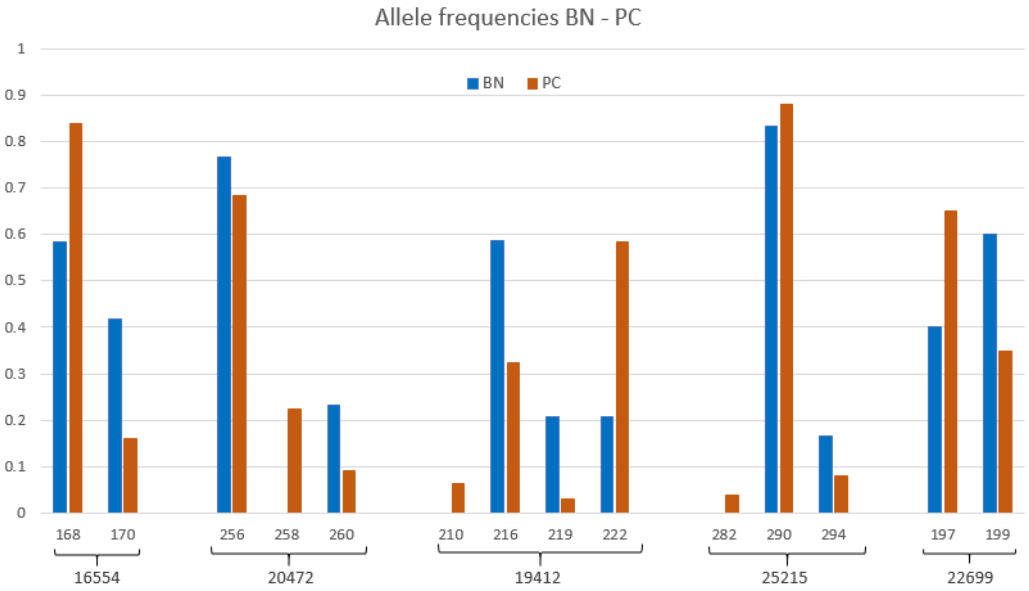
The table below (Table 7) shows all the alleles that were found for each microsatellite and their frequency within the Italian and European populations.

loci	alleles	Bosco Nordio	Porto Caleri	Arsago Seprio	Cameri	Ivrea	Croatia	Germany	Serbia
16554	150	0	0	0	0	0	0	0	0.0714
	156	0	0	0.0714	0	0.1818	0	0	0
	162	0	0	0	0	0	0.4375	0	0.4286
	166	0	0	0	0	0	0	0	0.0714
	168	0.5833	0.8400	0.9286	1	0.8182	0.3750	0	0.3571
	170	0.4167	0.1600	0	0	0	0.0625	0	0.0714
	172	0	0	0	0	0	0.1250	0	0
20472	254	0	0	0	0	0	0.1667	0	0.2500
	256	0.7679	0.6837	0.8571	1	1	0.8333	0	0.7500
	258	0	0.2245	0.1429	0	0	0	0	0
	260	0.2321	0.0918	0	0	0	0	0	0
19412	210	0	0.0625	0.1429	0	0	0	0	0
	213	0	0	0	0.0714	0	0.0556	0	0.1429
	216	0.5862	0.3229	0.5714	0.2857	0.5833	0.0556	0.2143	0
	219	0.2069	0.0312	0.1429	0.2143	0.4167	0	0	0.2857
	222	0.2069	0.5833	0	0.4286	0	0	0.6429	0
	226	0	0	0	0	0	0	0.0714	0
	228	0	0	0.1429	0	0	0	0	0.1429
	234	0	0	0	0	0	0.2222	0	0.0714
	236	0	0	0	0	0	0.0556	0	0.0714
	240	0	0	0	0	0	0.1667	0	0.2143
	242	0	0	0	0	0	0	0	0.0714
	246	0	0	0	0	0	0.2222	0.0714	0
	249	0	0	0	0	0	0.1111	0	0
	252	0	0	0	0	0	0.0556	0	0
	258	0	0	0	0	0	0.0556	0	0
25215	282	0	0.0400	0	0	0	0	0	0
	290	0.8333	0.8800	1	0.9286	1	1	0.0714	1
	294	0.1667	0.0800	0	0.0714	0	0	0.9286	0
22699	197	0.4000	0.6500	0.8571	1	1	0	1	0
	199	0.6000	0.3500	0.1429	0	0	1	0	0.7500
	210	0	0	0	0	0	0	0	0.2500
1509	186	0	0	0	0	0	0.1111	0	0.3333
	188	0	0	0.0714	0	0	0.1667	0.6429	0.8333
	192	0	0	0.1429	0	0.4167	0.1111	0.1429	0
	194	1	1	0.7857	1	0.5833	0.5000	0.2143	0.5833
	198	0	0	0	0	0	0.1111	0	0
7132	197	1	1	1	1	1	0.3889	0.3571	0.4286
	209	0	0	0	0	0	0.6111	0.5714	0.5714
	214	0	0	0	0	0	0	0.0714	0
11806	284	0.5000	0.5000	0.5000	0.500	0.4545	0.9444	0	0.2857
	286	0	0	0	0.2143	0.1818	0	1	0.5714
	288	0.5000	0.5000	0.5000	0.2857	0.3636	0.0556	0	0.1429

14420	**233**	0	0	0	0	0	0.1111	0	0
	235	0	0	0	0	0	0.7222	0.1429	0.6429
	237	1	1	0.6429	1	0.9583	0	0	0
	241	0	0	0.3571	0	0.0417	0.1667	0.5714	0.3571
	243	0	0	0	0	0	0	0.2143	0
	245	0	0	0	0	0	0	0.0714	0
19487	**243**	1	1	1	1	1	0.1667	0.1000	0.3571
	245	0	0	0	0	0	0	0.7000	0.3571
	247	0	0	0	0	0	0	0.2000	0.2857
	257	0	0	0	0	0	0.8333	0	0
18490	**286**	0	0	0	0	0	0	0.6667	0
	288	0	0	0	0	0	0	0.3333	0
	297	0	0	0	0	0	1	0	1
	299	1	1	1	1	1	0	0	0
18843	**261**	0	0	0	0	0	0.2500	1	0.0714
	263	1	1	1	1	1	0.5000	0	0.7143
	265	0	0	0	0	0	0.2500	0	0
	276	0	0	0	0	0	0	0	0.1429
	288	0	0	0	0	0	0	0	0.0714

(**Table 7**: Frequencies of all the alleles scored in 12 genotyped loci for 8 populations of different geographic areas. Only 1 out of the 11 genotyped siblings from BN was considered. When the value "1" is present, it indicates that the locus is monomorphic, that is, it has only that particular allele. When is “0” the allele is not present. Differently, the locus is polymorphic.)

Focusing on the population of Bosco Nordio, although at a first analysis the pattern of allelic presence shown in Table 7 is, as expected, very similar between the populations of BN and PC, compared to the other populations analysed. Going into the details of the allele frequencies at the different loci in these populations, a poor correspondence is observed. The allele frequencies are significantly different between the two populations, as evidenced by the significance of the locus-by-locus FST afterwards (Table 8). In order to visualize this difference, the allele frequencies at the 5 polymorphic loci of PC and BN have been visualized in the histogram in Fig.15.



(**Fig.15**: The histogram compares the allele frequencies of polymorphic loci for BN and PC populations.).

In conclusion, taking only two spawn to establish a new population in the BN Natural Reserve have caused an alteration of the original allele frequencies and the loss of 3 out of 14 original alleles.

To give significance to the difference in allele frequencies between BN and PC, FST with relative p-values between the two populations were calculated through a locus-by-locus AMOVA (Table 8).

	Bosco Nordio	Porto Caleri
locus	16554	
N	30	50
Na	2	2
FST / p-value	0.14704 / 0.00030	
locus	20472	
N	28	49
Na	2	3
FST / p-value	0.06916 / 0.00465	
locus	19412	
N	29	48
Na	3	4
FST / p-value	0.16908 / 0.00000	
locus	25215	
N	30	50
Na	2	3
FST / p-value	0.00978 / 0.19901	

locus	22699	
N	30	50
Na	2	2
FST / p-value	0.10680 / 0.00248	
locus	11806	
N	30	50
Na	2	2
FST / p-value	-0.01351 / 1.00000	

(**Table 8**: Indices of differentiation between BN and PC locus by locus. "N" represents the number of genotyped individuals, "Na" the number of alleles for the given locus. FST fixation indices and relative significance (p-value) are calculated. Loci 16554, 20472, 19412 and 22699 have significant p-value (<0.05).)

4.2.3. POPULATION DIFFERENTIATION

A hierarchical AMOVA was performed from the information obtained in the matrix of FST, where the number of different alleles is used as distance method in pairwise populations (Table 9). The dataset was composed by 129 individuals from Italian and extra-Italian populations.

	Bosco Nordio	**Porto Caleri**	**Arsago Seprio**	**Cameri**	**Ivrea**	**Croatia**	**Germany**	**Serbia**
Bosco Nordio		0.00000	0.00020	0.02841	0.00000	0.00000	0.00000	0.00000
Porto Caleri	0.07460		0.00000	0.04861	0.00000	0.00000	0.00000	0.00000
Arsago Seprio	0.10266	0.19836		0.00752	0.03752	0.00030	0.00059	0.00069
Cameri	0.04338	0.01966	0.11815		0.01049	0.00020	0.00040	0.00089
Ivrea	0.14054	0.25509	0.08089	0.13452		0.00000	0.00000	0.00000
Croatia	0.67340	0.70350	0.53282	0.58061	0.60081		0.00000	0.00772
Germany	0.75515	0.77497	0.65241	0.67757	0.69955	0.53537		0.00059
Serbia	0.62368	0.66363	0.43929	0.48628	0.51749	0.12971	0.44273	

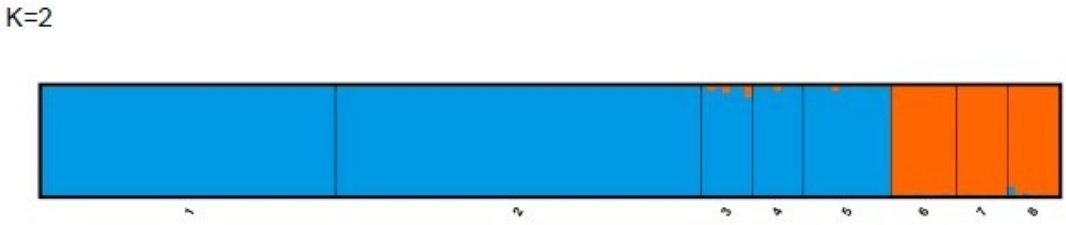
(**Table 9**: Fixation index value (FST) matrix between pairs of *Pelobates fuscus* populations based on 12 loci (below the diagonal) and relative p-value (above the diagonal). Only 1 out of the 11 genotyped siblings from BN was considered. All pairs are found to have significant p-value even after correction for multiple Bejamini-Hochberg tests.).

FST value among populations are reported in Table 9; all values are significant even after Benjamini-Hochberg correction for multiple comparisons. Remarkably high FST are observed in the comparisons between Italian and non-Italian populations confirming a very high differentiation. On contrary, the comparisons among Italian populations show a drastic decrease of the genetic structure. In particular, for BN

and PC the values are very low, as expected given the common origin, but significant, clearly showing that the translocated individuals were not representative of the original genetic composition of the source population. The low values observed in the comparisons with the population of Cameri are also low but the very low number of individuals of this location make this result poorly reliable.

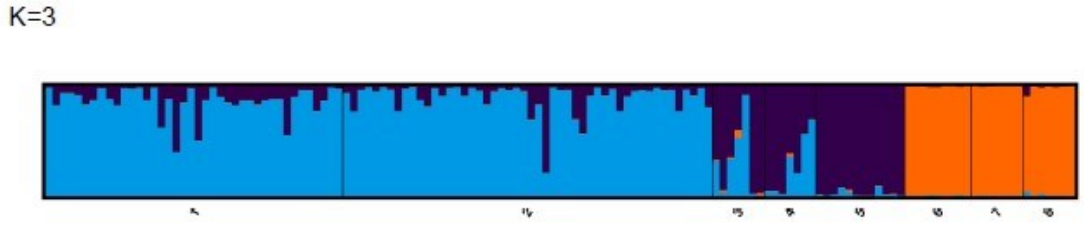
In order to test the ability of microsatellites to subdivide the species of interest into populations, a clustering analysis was carried out with Structure software (Pritchard et al., 2000) followed by StructureHarvester (Earl & vonHoldt, 2011) and Clumpak (Kopelman et al., 2015).

Figure 16 shows the result of the run performed from the dataset consisting of the genotype of 139 individuals (Italian and non-Italian) for 12 selected loci. For group structuring, the program selected K=2 as the more likely.



(**Fig.16**: Structure bar-plot showing group analysis for K=2. Numbers from 1 to 8 correspond to the populations in the order: Bosco Nordio, Porto Caleri, Arsago Seprio, Cameri, Ivrea, Croatia, Germany, and Serbia. The two identified groups are represented by two different colours.)

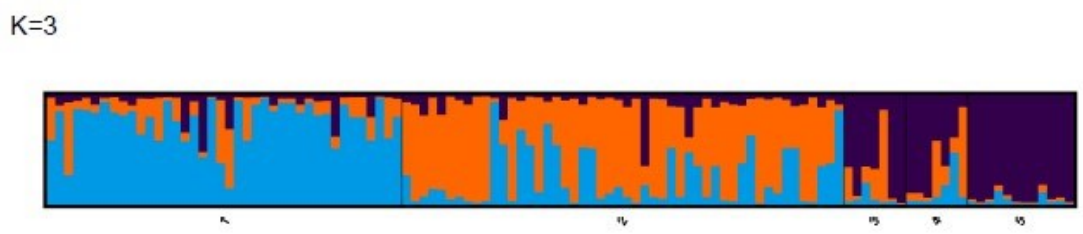
The distinction between Italian and foreign populations previously observed with mitochondrial DNA is clearly confirmed by microsatellites.



(**Fig.17**: Structure bar-plot showing group analysis for K=3. Three groups are identified by three different colours. Individuals within the plot are represented by a single vertical bar. The colour of each vertical bar represents the probability that the given individual belongs to the identified groups.)

Although not chosen as the best, also K=3 (Fig.17), differentiates the group formed by BN (1) and PC, (2) from the other Italian populations (3, 4 and 5), also confirming the distinction of Croatia, Germany, and Serbia from the Italian cluster.

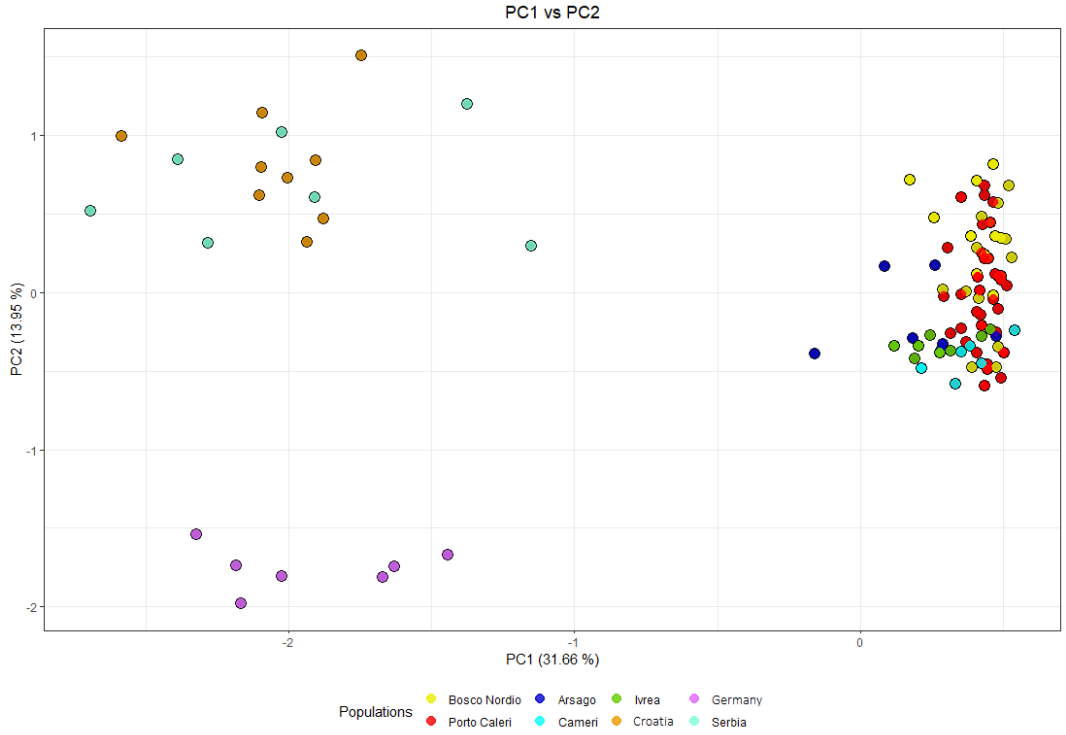
By using only the genotypic data of Italian populations for the analysis, K equal to 3 is chosen as the best (Fig.18).



(**Fig.18**: Structure bar-plot showing group analysis for K=3. The dataset was formed only by Italian samples).

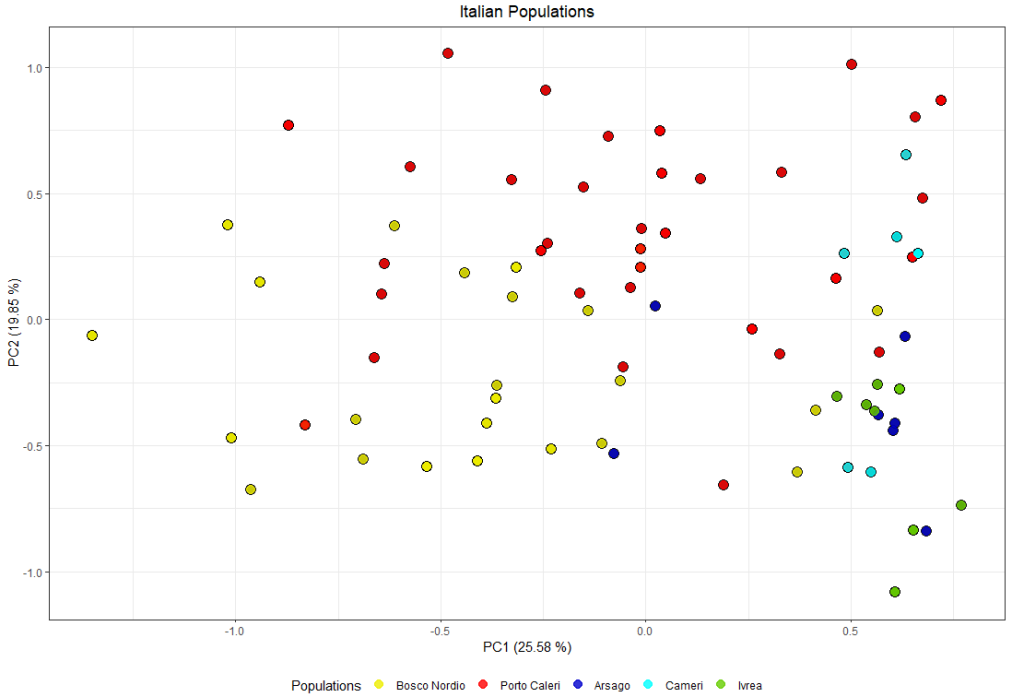
Within the Italian context, there is an additional separation between BN and PC. The third cluster consists instead of Arsago Seprio, Cameri and Ivrea, which are grouped together.

To evaluate the clustering division with another method and estimate the distances between the groups, RStudio (R core team, 2017) package was used to perform two PCA. A first graph was produced using 139 individuals from Italian and extra-Italian individuals for 12 loci. On the x-axis of the principal components plot (Fig.19), PC1 explains 31.66 percent of the variance and confirm the clear separation of Italian populations from other European populations, as previously seen in the structure analysis. On the y-axis PC2 explains 13.95 percent of the variance and exhibits a distinction between a Serbian-Croatian group and a German one. This gene-level clustering effectively reflects the geographic-level separation of the populations in analysis.



(**Fig.19**: Principal Component Analysis plot for PC1 and PC2 in 139 samples for 12 microsatellite loci. Each colour corresponds to a given population. Italian populations cluster on the right, while European populations on the left of the graph.)

To go into more detail, a second PCA was performed by entering data from 116 individuals with Italian origin, for the same 12 microsatellite loci genotyped (Fig.20). There is no obvious clustering in the plot, but it is possible to see a slight separation between the populations of Arsago Seprio, Cameri, and Ivrea, shifted to the right side of the graph, with those of BN and PC, in to the center-left, along the axis of PC1. While on the PC2 axis a low-level division between the BN and PC populations is observed.



(**Fig.20**: Principal Component Analysis plot for PC1 and PC2 in 116 Italian samples for the same 12 microsatellite loci. PC1 explains 25.58 percent of the total variance while PC2 explains 19.85 percent.).

The intra-species segregation in the Italian territory observed in (Fig.18-20), it is probably due to the creation of reproductively isolated populations of *Pelobates fuscus* in different parts of the Po Valley. The nature of the segregation is unclear, but since the species has a fossorial lifestyle and low dispersal ability, once established in a suitable environment, it is unlikely to migrate to other populations, especially if not connected by viable corridors for the animal. In the Po Valley, urbanization is marked and heavily affects the land, plus agriculture is often intensive. These factors change the environment leading to the disappearance of the *spadefoot toad* at many sites or precisely by favouring the stabilization of specimens in a disconnected area (Andreone et al., 2004). If populations consist of only a few individuals, the likelihood of mating between siblings is higher potentially leading to inbreeding depression. This phenomenon can lead to reduced survival or fertility of subsequent generations due to increased homozygosity (Charlesworth & Willis, 2009).

Taking into consideration the two populations of greatest interest for this study, Bosco Nordio and Porto Caleri populations, since the Bosco Nordio population was created from two spawn taken in the wild in Porto Caleri (Richard & Vianello, 2016), the differentiation between these two samples suggests that in the reintroduction process the source population was not properly represented, given the very limited number of animals transferred, as was somehow expected. This

resulted in a population bottleneck, that causes a loss of allelic diversity and heterozygosity, in which rare alleles are the ones that are lost the most (Fig.15) (Hundertmark & Van Daele, 2009).

5. CONCLUSION

Despite the problems encountered in the analysis due to the low variability of the microsatellite loci, the small number and low quality of samples, it was possible to highlight how the Bosco Nordio and Porto Caleri populations fit consistently among other Italian populations, but showing their own genetic identity. The origin of the differentiation is unknown, perhaps it is caused by a founder effect and genetic drift given by small population size and isolation.

The reintroduced population of Bosco Nordio shows obvious genetic erosion compared to the source population of Porto Caleri, which is already little variable in itself. On this basis, management decisions will need to be taken, to promote the conservation of the populations of interest. Regarding Bosco Nordio, further translocations will be necessary so as to increase the genetic variability of individuals. As for the Porto Caleri population, if the effects of inbreeding lead to the appearance of malformed specimens and a decline in fertility, it will be necessary to consider the possibility of translocating individuals from other geographic areas.

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