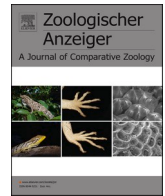


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## Phylogeography and population genetics of the endemic Italian water voles, *Arvicola italicus*

Emanuela Solano<sup>a,b</sup>, Riccardo Castiglia<sup>c,\*</sup>, Giovanni Amori<sup>a</sup>, Giulia Gentile<sup>a</sup>, Sandro Bertolino<sup>d</sup>, Dario Capizzi<sup>e</sup>, Petra Kranebitter<sup>f</sup>, Eva Ladurner<sup>f</sup>, Luca Lapini<sup>g</sup>, Paolo Colangelo<sup>a,b</sup>

<sup>a</sup> National Research Council, Research Institute on Terrestrial Ecosystems, Montelibretti, Rome, Italy

<sup>b</sup> National Biodiversity Future Center (NBFC), Palermo, Italy

<sup>c</sup> Department of Biology and Biotechnology "Charles Darwin", Sapienza University, Rome, Italy

<sup>d</sup> Department of Life Sciences and Systems Biology, University of Turin, Turin, Italy

<sup>e</sup> Regione Lazio – Agenzia Regionale per i Parchi, via del Pescaccio 96, 00166, Rome, Italy

<sup>f</sup> Naturmuseum Südtirol - Museo di Scienze Naturali dell'Alto Adige, Bolzano, Italy

<sup>g</sup> Friulan Museum of Natural History, Udine, Italy

### ARTICLE INFO

Handling Editor: Janine M Ziermann

#### Keywords:

Microsatellite  
MtDNA  
Taxonomy  
Refugia  
Ecotype

### ABSTRACT

The genus *Arvicola* has a wide palearctic distribution and present two ecological forms – terrestrial and aquatic – characterizing the genus. In Europe two species are present: the most widespread, *A. amphibius* and *A. italicus*, endemic to Italy. The aim of the study was to describe the genetic structure and variability in *A. italicus* and the genetic identity of populations in northeast Italy, an area of biogeographic importance as contact zone for small mammal species.

Thirty-six Italian water voles, displaced in population from south to north of Italy, were analysed by two mitochondrial markers and 8 autosomal microsatellite loci. The phylogenetic analysis on the genus confirmed the presence of major groups mostly corresponding to the species and clades already described. About the Italian specimens, the individuals from one population in northeastern Italy resulted included in *A. amphibius* revealing the presence of this species in Italy and, in consequence, the possible presence of a putative contact zone between *A. italicus* and *A. amphibius* in the northeast. The phylogeny includes a second population from northeast Italy within *A. amphibius*, but in basal position and with a low support. The values of divergence of this lineage from *A. italicus* and *A. amphibius* lie on the threshold between intraspecific and interspecific divergence. Conversely, microsatellite data support a clear distinction between *A. amphibius* and *A. italicus* and includes the ambiguous mitochondrial lineage in this latter species. Furthermore, this northeastern population shows evidence of admixture of nuclear loci with the southcentral *A. italicus* populations, supporting the attribution of these specimens to *A. italicus*. This pattern could suggest that *A. italicus* in northeast Italy retained an ancestral haplotype and undergone a long period of isolation respect to the southern populations, without genetic exchange until recent times. Within *A. italicus*, the admixture shows northern population as the most differentiated showing limited evidence of admixture with the most diverse southcentral. The intraspecific diversity decreases from south to north as expected for an endemic small mammal in Italy. Finally, the low genetic diversity observed in the northern population can be challenging for a species as *Arvicola* living in fragmented habitats and susceptible to habitat loss; consequently, became important identifying suitable conservation measures for this barely known endemic small mammal.

\* Corresponding author.

E-mail addresses: [emanuela.solano@cnr.it](mailto:emanuela.solano@cnr.it) (E. Solano), [riccardo.castiglia@uniroma1.it](mailto:riccardo.castiglia@uniroma1.it) (R. Castiglia), [giovanni.amori@cnr.it](mailto:giovanni.amori@cnr.it) (G. Amori), [giuliagentile96@gmail.com](mailto:giuliagentile96@gmail.com) (G. Gentile), [sandro.bertolino@unito.it](mailto:sandro.bertolino@unito.it) (S. Bertolino), [dcapizzi@regione.lazio.it](mailto:dcapizzi@regione.lazio.it) (D. Capizzi), [petra.kranebitter@naturmuseum.it](mailto:petra.kranebitter@naturmuseum.it) (P. Kranebitter), [esm.ladurner@gmail.com](mailto:esm.ladurner@gmail.com) (E. Ladurner), [luca.lapini@comune.udine.it](mailto:luca.lapini@comune.udine.it) (L. Lapini), [paolo.colangelo@cnr.it](mailto:paolo.colangelo@cnr.it) (P. Colangelo).

<https://doi.org/10.1016/j.jcz.2024.09.003>

Received 31 May 2024; Received in revised form 22 September 2024; Accepted 30 September 2024

Available online 2 October 2024

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## 1. Introduction

The taxonomic status of the water voles of the genus *Arvicola* Lacépède, 1799 have been investigated in the last years through several approaches based on morphology, molecular DNA and chromosomes (Castiglia et al., 2016; Chevret et al., 2020; Kryštufek et al., 2015; Mahmoudi et al., 2019; Musser and Carleton, 2005). The genus has a wide (Palearctic) distribution (from Western Europe to Siberia), a complex phylogeographic pattern and shows two clearcut different ecological form – i.e. ecotypes - fossorial and aquatic. Particularly, the presence of two different ecotypes makes taxonomy of the genus challenging.

The most recent molecular phylogenetic and morphological studies for the European species report the presence of three or four distinct species (Brace et al., 2016; Castiglia et al., 2016; Chevret et al., 2020; Kryštufek et al., 2015; Kryštufek, 2017). The aquatic *Arvicola sapidus*, is present in the Iberian Peninsula and in France (Brace et al., 2016). The most widespread, *A. amphibius* comprehends two genetically distinct clades: the Euroasiatic clade (EU) distributed from Europe to western Siberia with both aquatic and fossorial ecotypes and the Western-European clade (WE) described in Switzerland and Spain with strictly fossorial/montane habit. The WE clade was treated as a distinct species, *A. monticola*, by Mahmoudi et al. (2019) but this distinction is not accepted by Chevret et al. (2020) and by Kryštufek et al. in the last revision (2022). Castiglia et al., in 2016 focused their study on Italian populations of water voles and confirmed the presence in Italy of a clade, genetically divergent in *Arvicola* phylogeny, that can be attributed to a distinct species, *Arvicola italicus* Savi, 1838. This species is nearly endemic to the Italian peninsula even if individuals from Switzerland (locality Lucerne, which is close to the Italian border) belongs to this lineage (Brace et al., 2016). According to the Italian species red list, *A. italicus* is categorized as data deficient (DD, Rondinini et al., 2022). This species is often associated with fragmented habitats, which may be subject to habitat loss (Dean et al., 2016; Jeffries et al., 1989) or threatened by competition with allochthonous species (i.e. American mink; Bertolino et al., 2023; Loy et al., 2019; Mori and Mazza, 2019), especially in aquatic habitat. The mitochondrial DNA analysis of *A. italicus* revealed the presence of two separate lineages that correspond to the north and to the southcentral part of the Peninsula, showing a certain variability among Italian populations. In this species, the aquatic ecotype shows a wide distribution in most of the peninsula while the strictly fossorial ecotype was principally reported from mountain areas in the north but was also found in lowland orchards; it is worthy to mention that no phylogenetic distinction between the two forms is present (Castiglia et al., 2016). In this paper, following the last revision, we will consider *A. amphibius* and *A. italicus* as a good distinct species.

It is well established the influence of quaternary climate oscillations on the evolution of European biota and, in turn, on the intra and interspecific diversification of small mammals. For *A. amphibius*, all the authors agree with a postglacial colonization pattern with the presence of Southwest Asian (Mahmoudi et al., 2019), Iberian and Northeastern continental (Brace et al., 2016; Chevret et al., 2020; Piertney et al., 2005) refugia. The same authors showed that in *A. amphibius*, the WE lineage (*A. monticola* in Mahmoudi et al., 2019) widespread in central Europe at the beginning of the last glaciation (Pleistocene), survived during the LGM (Last Glacial Maximum) in the Iberian refugia. Conversely, the EU lineage retreated in glacial refugia in North Europe and expanded in the temperate period after Younger Dryas (early Holocene: 12000bp-4500bp) defining the nowadays distribution of the two lineages (Brace et al., 2016; Piertney et al., 2005). *A. italicus* originated in consequence of long-term isolation within the Italian peninsula and was unable to cross the Alps to the north, and as a result, its differentiation followed a different course. Moreover, several studies evidenced the possible occurrence of climate-driven cycles of allopatric differentiation within refugia themselves, the so called ‘refugia within refugia’ scenario (Gómez and Lunt, 2007). In fact, a growing number of taxa

reveal evidence of strong phylogeographic structure within the main Mediterranean peninsulas (Centeno-Cuadros et al., 2009; Dubreuil et al., 2008; Ferrero et al., 2011): in the Iberian (Canestrelli et al., 2008; Canestrelli et al., 2014), in the Italian (Hardion et al., 2014; Pabijan et al., 2015; Ursenbacher et al., 2008) and in the Balkans (Deffontaine et al., 2005).

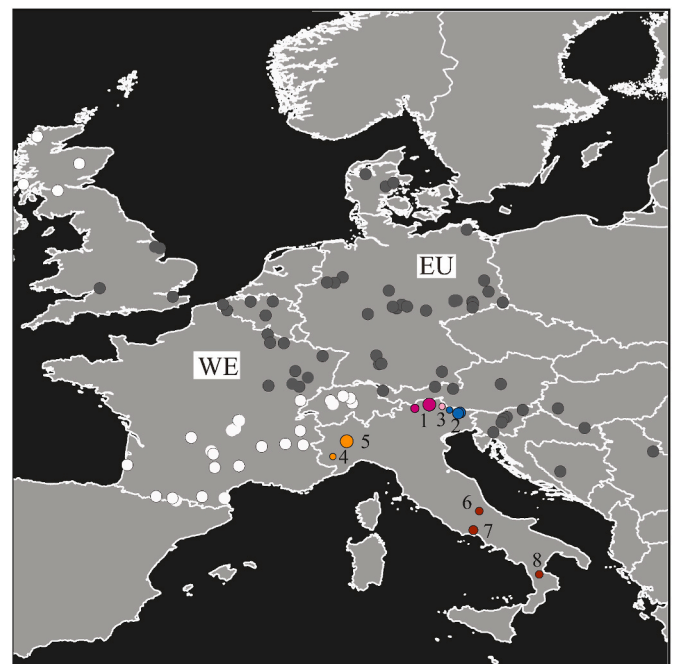
All those previous studies open interesting questions about the intraspecific variability of *A. italicus* and taxonomic identity of the populations inhabiting marginal areas of Italy. This points out the necessity to investigate the populations inhabiting the northern part of the peninsula. The aim of the present study was to investigate, by mitochondrial and nuclear molecular markers, to investigate the genetic identity of populations in north Italy, an area of extreme biogeographic importance (Schmitt et al., 2021). This area also represents a potential contact zone between *A. italicus* and *A. amphibius*. Additionally, the study aimed to describe the genetic structure and variability within *A. italicus*.

## 2. Materials and methods

### 2.1. Sample

A total of thirty-eight specimens were used in this study. Twenty were new samples whereas the remaining 18 were previously analysed for cytochrome *b* (*CYTB*) by Castiglia et al. (2016).

The 20 new vole’s specimens come from the Northeastern part of Italy: eleven from Trentino Alto Adige (population code: TAA; Bolzano province), one from Veneto region (population code: VEN; Belluno province), 7 from Friuli-Venezia Giulia region (Udine province) plus one from Carinzia (Austria), close to the Italian border (population code: FVG). See map in Fig. 1 and Table 1 for details and distribution of the sample. All these specimens show a terrestrial/fossorial ecotype and were sampled during previous campaigns by means of live traps placed at the entrance of the openings to their burrows. The sample size is not



**Fig. 1.** Distribution map of the sample of *A. amphibius* and Italian populations from present and previous studies. The Western European and Euroasiatic clades of *A. amphibius* are reported in white (WE) and black (EU) dots respectively. The Italian new and previous sample are in coloured dots and the size of the dots is proportioned to the number of samples for each population. The colours follow Fig. 2 and the numbers refer to the localities in Table 1.

**Table 1**

Overview of the sample analysed. Sample locality reference number on map (Map), number of specimens per population (N°), population code (Code), sample locality details (Province and Locality), voucher of sampled specimens (Voucher), mitochondrial DNA lineage (MtL) and ecotype are reported. For each individual is reported the type of sequences obtained: (\*) microsatellite, (a) CYTB, (b) CR. In bold the new samples from this work.

Map	N°	Region	Code	Province	Locality	Voucher *microsatellite/ <sup>a</sup> CYTB/ <sup>b</sup> CR	MtL	ecotype
1	11	Trentino Alto Adige	TAA	Bolzano	Brunico	<b>BZ01<sup>*,a,b</sup>, BZ02<sup>*,a,b</sup>, BZ03<sup>*,a</sup>, BZ04<sup>*,a</sup>, BZ05<sup>*,a,b</sup>, BZ06<sup>*,a,b</sup>, BZ07<sup>*,a,b</sup>, BZ08<sup>*,a,b</sup></b>	EU	Fossorial
				Bolzano		<b>BZ10<sup>a</sup>, BZ11<sup>a,b</sup>, BZ12<sup>a,b</sup></b>	EU	Fossorial
2	8	Friuli Venezia Giulia	FVG	Udine	Tarvisio	<b>TA00<sup>*,a,b</sup>, TA02<sup>*,a,b</sup>, TA03<sup>*,a</sup>, TA04<sup>*,a,b</sup>, TA05<sup>*,a</sup>, TA08<sup>*</sup></b>	NE	Fossorial
		Austria		Carinzia	Oberthoerl	<b>TA01<sup>*,a</sup></b>	NE	Fossorial
				Udine	Rigolato	<b>TA07<sup>*,b</sup></b>	NE	Fossorial
3	1	Veneto	VEN	Belluno	Belluno	<b>VEN<sup>*,a</sup></b>	NE	Fossorial
4	8	Piedmont	PIE	Cuneo	Verzuolo	PIEL 1 <sup>a,b</sup> , PIEL 2 <sup>a,b</sup> , PIEL 3 <sup>*,a,b</sup> , PIEL 4 <sup>*,a</sup> , PIEL 5 <sup>*,a,b</sup> , PIEL 6 <sup>*,a,b</sup> , PIEL 7 <sup>*,a,b</sup> , PIEL 8 <sup>*,a,b</sup>	NW	Fossorial
5	2	Piedmont		Vercelli	S. Genuario	A-PIEL <sup>*,a,b</sup> , B-PIEL <sup>a,b</sup>	NW	Aquatic
6	2	Abruzzo	POP	Pescara	Popoli	POP A <sup>*,a,b</sup> , POP B <sup>*,a,b</sup>	SC	Aquatic
7	4	Latium	FON	Latina	Fondi	FON 1 <sup>a,b</sup> , FON 2 <sup>a,b</sup> , FON 3 <sup>a,b</sup> , FON 4 <sup>a,b</sup>	SC	Aquatic
8	2	Calabria	CAL	Cosenza	Sila Grande	CAL1 <sup>*,a,b</sup> , CAL2 <sup>*,a,b</sup>	SC	Aquatic

large because the species is not easy to sample in Italy due to its highly localized populations and specific habitat. These specimens are deposited at the Museo di Scienze Naturali dell'Alto Adige - Naturmuseum Südtirol (Bolzano) and the Friulan Museum of Natural History (Udine). The DNA of the 18 specimens from Castiglia et al. (2016) was available at the Department of Biology and Biotechnologies of the University of Rome "La Sapienza". Details are reported in Table 1.

The DNA of the 20 new samples was extracted according to the salting out procedure (Aljanabi and Martinez, 1997). For the most difficult samples the QIAmp genomic DNA extraction kit (Quiagen) was used following the manufacturer's procedure.

## 2.2. Phylogeny and phylogeography

The cytochrome *b* (CYTB) mitochondrial gene were successfully isolated on 20 individuals with the universal primers L14723 and H15915 described in Irwin et al. (1991) under the standard PCR conditions. The D-loop Control Region (CR) were successfully isolated on 29 individuals (12 new and 17 samples from Castiglia et al., 2016) with the primers F15708 and R92 following the protocol used in Piartney et al. (2005). The gene amplifications were performed in a Biometra Thermocycler. Both forward and reverse primers for each gene were used to sequence the fragments in double strand. All the sequences were inspected by eye, eventually manually edited, and aligned using Geneious v. 9 (Biomatter). The inspection of the CYTB sequences does not reveal the presence of stop codons. All the new sequences have been deposited in GenBank (Accession numbers PQ429244-PQ429286; Appendix Table A.1).

For the phylogenetic analysis, a CYTB dataset have been created, composed by 300 total samples (alignment of 1140bp): 36 individuals from Italy, 260 GenBank sequences including sequences of European species *A. sapidus*, *A. amphibius* plus sequences of the recently described species *A. persicus* from Iran (Barbosa et al., 2013; Chevret et al., 2020; Fink et al., 2010; Krystufek et al., 2015; Mahmoudi et al., 2019) and 4 outgroups (*Myodes glareolus* AM392368, *Eothenomys melanogaster* AM392374, *Ellobius tancrei* MK544900 and *Microtus arvalis* AM991045).

The Bayesian Inference (BI) approach was used to construct the phylogenetic tree on the CYTB matrix. Firstly, the best substitution model for the CYTB dataset at each codon position was evaluated using the JModelTest (Posada, 2008) under the AIC criterion.

We considered the CYTB as a single partition since the analysis in JModelTest suggested to apply the Generalized Time-Reversible Model with a proportion of invariable sites and heterogeneous substitution rates following a gamma distribution (GTR + I + G, Rodríguez et al.,

1990) to all the three codon positions. To avoid over-parameterization we applied the model to entire dataset without partition (Guimarães and Höhna, 2023).

The BI analysis was carried out with MrBayes v. 3.2.1 (Huelsenbeck and Ronquist, 2001) by running 10,000,000 generations, with Markov chains sampled every 1000 generations. A burn-in of 10 % was applied and the remaining trees were used to compute a 50 % majority rule consensus tree and posterior probabilities. Convergence was checked using the software Tracer v.1.7 (Rambaut et al., 2014) and the burn-in was graphically determined. On the same dataset Maximum Likelihood (ML) analysis was performed using IQ-TREE (Nguyen et al., 2015), implemented in the web server version (<http://iqtree.cibiv.univie.ac.at/>) (Trifinopoulos et al., 2016). The robustness of the nodes was assessed by bootstrap with XXXX replicates. Pairwise genetic divergence among the major clades emerged from the phylogenetic analysis (see results) was calculated using the Kimura 2-parameter model (K2P distances between group) as implemented in MEGA 11 (Kumar et al., 2016). The standard molecular diversity index *o*, number of haplotypes (*h*), number of polymorphic sites, and nucleotide ( $\pi$ ) and haplotype (Hd) diversity were calculated for the *A. italicus* lineages resulting from BI analysis (see results) with the software DNASP v.6 (Librado and Rozas, 2009).

The Italian CYTB sequences have been joined with the new CR sequences of the same samples on a combined data matrix to increase the polymorphic sites and then the phylogenetic signal. We obtained a dataset composed by 28 specimens for which both genes were sequenced that was subsequently used to build a Median Joining network using the POPART software (Leigh and Bryant, 2015) in default settings to show the relationships among haplotypes. This method was chosen because provides the best estimate of the true genealogy when internal node haplotypes are not sampled (Cassens et al., 2005).

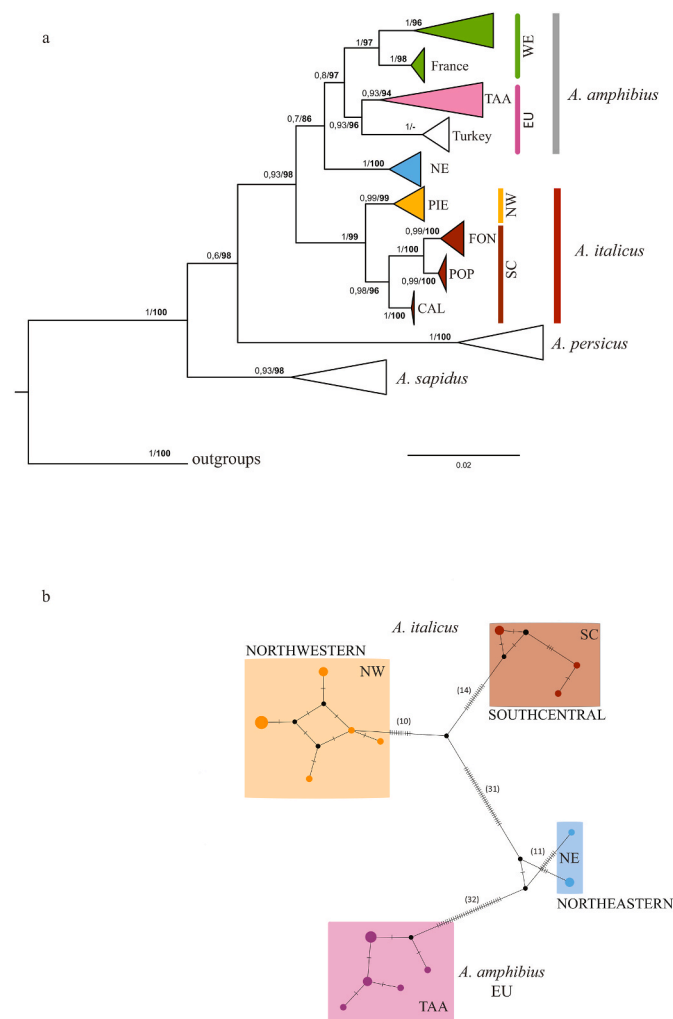
## 2.3. Population genetics

On a subset of 27 Italian individuals, multilocus genotypes were obtained by amplification of a panel of 8 autosomal microsatellites loci (modified from Stewart et al., 1998) divided into two mixes: mix1 (AV9, AV10, AV3, AV15) and mix2 (AV11, AV12, AV8, AV14). For each mix the forward primers were labelled at the 5'-end with different fluorescent dye groups (6-FAM, Hex, Rox, Tamra) (details in Table 2). The amplifications of the different mixes were performed as follows: 94 °C for 2 min and 15 sec, 45 cycles at 94 °C for 30 sec, 55–61 °C for 30 sec (with different annealing temperatures depending on the mix), 72 °C for 30 sec, and a final extension at 72 °C for 5 min. All microsatellite loci

**Table 2**

Primer sequences for each of the eight loci. Name, sequences, fluorescent label, range of amplification and mix used are reported. See text for further details.

Oligo name	Forward	Reverse	5'-mod	range	mix
AV10	AACTGCTGAGCATCTCCAGAC	TCAGGGCTAGGATATACTACATAAATC	6-FAM	162–187	1
AV9	CACTGGCTCAGATTCAAGACTAC	AGGGAGGAAAAGCTAGGTCACAG	HEX	219–329	1
AV15	TATATGGAAGTTCGTAGATTTCAG	ATTAAAGCATTGTTGAGAAAGC	ROX	172–216	1
AV3	GGATCAACCAGGTCCAGCAC	AAAGAGCTAGTGGGATTCCTAAG	TAMRA	126–159	1
AV11	TGGCCTTATCAGGAAACATAC	GAATAGCTTGCTCTGATGGC	6-FAM	280–392	2
AV12	GAGGCAGGAGGATAACAAG	CCATCTCTCAAATCCATGAC	HEX	308–341	2
AV14	TATGTGATATGGCACTAGCATGT	AGCCTGTCTCAGCAGAAGG	ROX	194–294	2
AV8	GGGACAGAAGGAAGTAGAGG	GCAGTGGTAACAAGAGGATG	TAMRA	292–328	2



**Fig. 2.** Molecular phylogenetic analysis (a) The CYTB BI phylogenetic 50 % majority rules consensus tree. The BI posterior probability  $p > 0.5$  and the bootstrap values from the ML analysis are reported at nodes. Names of the species, major clades and lineages names are reported aside. See the text for details. (b) The MJN analysis on combined (CYTB and CR data, see text for details) Italian dataset. The colour pattern follows Figure (1 and 2a).

considered showed polymorphism and were used for the genotyping.

Genotyping was carried out with the ABI 3130 biosystem and the results were visualized with Peak Scanner software application from Thermo Fisher Scientific (Applied Biosystems).

The Italian population used in the microsatellite analysis have been define on the basis of geography and on lineages emerged in mitochondrial tree: NW, SC, TAA and the northeastern Italian lineage (NE) (see results). The data were tested for the presence of null alleles to evaluate the potential excess of homozygotes. To investigate the genetic diversity, we analysed: (i) the allele frequencies (ii) the allele richness

(AR) (iii) expected ( $H_e$ ) and observed ( $H_o$ ) heterozygosity values for each population. For each locus, deviations from Hardy–Weinberg Equilibrium and Linkage Disequilibrium were calculated in all the populations using Genepop (Rousset, 2008) with default Markov chain parameters. The spatial pattern of genetic variability among population was represented using the spatial PCA (sPCA) as implemented in the R package adegenet (Jombart, 2008). To assess the level of admixture among individuals of *A. italicus*, we used the “Tess3r” R package testing K values from 2 to 10, where K is the number of ancestral populations and the cross-validation score for each k value was calculated.

**Table 3**  
The *CYTB* genetic divergence among the species of the genus *Arvicola* and the NE clade.

	<i>A. sapidus</i>	<i>A. persicus</i>	<i>A. amphibius</i>	<i>A. italicus</i>	NE	Outgroup
<i>A. sapidus</i>	–					
<i>A. persicus</i>	9.32	–				
<i>A. amphibius</i>	6.99	9.22	–			
<i>A. italicus</i>	7.61	8.59	4.53	–		
NE	6.45	9.15	3.71	4.40	–	
Outgroup	15.84	16.83	16.62	15.54	16.39	–

**Table 4**  
Diversity index calculated for northeastern (NE), southcentral (SC) and northwestern (NW) lineages and on the pooled sample of *Arvicola* in Italy.

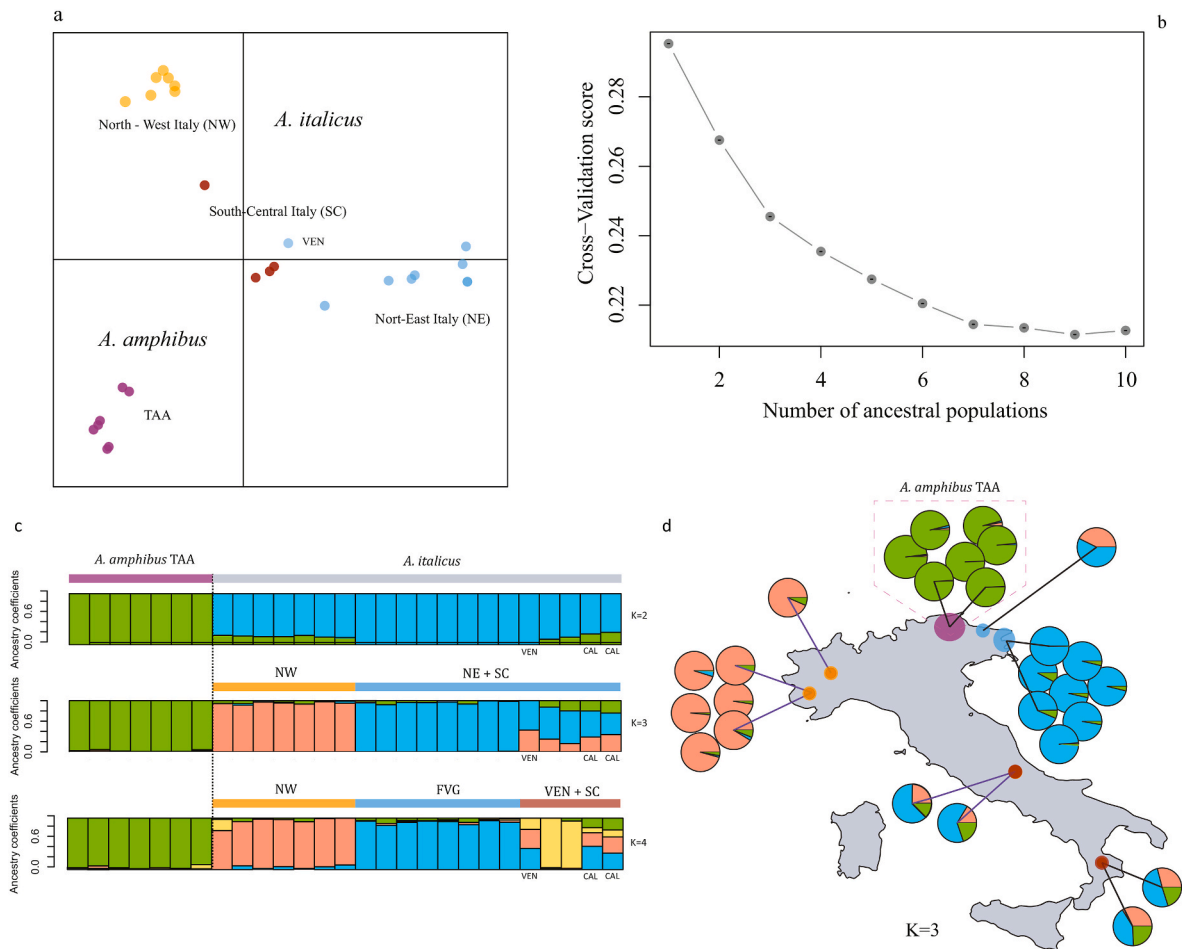
	$\pi$	$\theta$	<i>h</i>	Hd	variance	SD	PS	N.
NE	0.00688	0.00649	5	0.905	0.01067	0.103	13	817
NW	0.00117	0.00109	3	0.644	0.01025	0.101	2	647
SC	0.01098	0.00999	5	0.893	0.00736	0.086	16	618
NE + SC + NW	0.02547	0.01970	13	0.930	0.00086	0.029	45	605

**3. Results**

**3.1. Phylogeny and phylogeography**

Both phylogenetic trees based on *CYTB* show identical topology. In Fig. 2, we present the phylogenetic tree from the Bayesian analysis, with both posterior probability (p.p.) and bootstrap values (b.) indicated at the nodes. The *CYTB* phylogenetic trees (Fig. 2a) show that the genus

*Arvicola* is monophyletic (p.p. = 1/b. = 100). Four main well supported clades are present within the genus, corresponding to *A. sapidus* (p.p. = 0.93/b. = 98), *A. persicus* (p.p. = 1/b. = 100), *A. amphibius* (p.p. = 0.8/b. = 97) and *A. italicus* (p.p. = 1/b. = 99). Within *A. amphibius* we found two clades corresponding to the WE (p.p. = 1/b. = 97) and EU (p.p. = 0.93/b. = 96) clades (Brace et al., 2016; Castiglia et al., 2016; Chevret et al., 2020; Kryštufek et al., 2015; Kryštufek, 2017). In the EU clade two well supported lineages are present. One lineage includes the



**Fig. 3.** Microsatellite analysis (a) Spatial PCA (sPCA). The color of lineages and individuals is the same of Fig. 2a and b; (b) Cross validation test; (c) Admixture analysis. Only K values from 2 to 4 are reported; (d) geographic representation of probability distribution of ancestral population structure (K = 3). The colour pattern follows Figure (1 and 2a, 2b).

three Turkish specimens as previously described in [Chevret et al. \(2020\)](#), while the second lineage is widely distributed in eastern Europe, encompassing all the new individuals from TAA population (Trentino Alto Adige, Northern Italy).

Within the *A. italicus* clade, we found two distinct lineages corresponding to the northwestern and southcentral peninsular populations (called NW and SC respectively). Finally, a previously undetected clade (p.p. = 1/b. = 100), including the voles from northeastern Italian sample (all FVG samples plus VEN individual named NE hereafter), was detected. This latter clade shows uncertain phylogenetic position since it is in sister relationships with *A. amphibius* but with only moderate support (p.p. = 0.7/b. = 86).

The *CYTB* genetic divergence ([Table 3](#)) among the species of the genus *Arvicola* range from 4.5 % (value of divergence between *A. amphibius* and *A. italicus*) to 9.3 %. The divergence between the *A. amphibius* clades (EU and WE) is 3.9 % (not reported in [Table 3](#)). The NE clade shows higher divergence with *A. sapidus* (6.4 %) and *A. persicus* (9.1 %) than from *A. amphibius* and *A. italicus* (3.7 % and 4.4 % respectively).

The results of the diversity index calculated for NE, SC and NW lineages and on the pooled sample are reported in [Table 4](#). The highest  $\pi$  was found in the SC lineage (0.0109) and in the pooled sample (0.0255) while a lower value was found in NE (0.00688) and NW (0.00117). The highest Hd was found in NE (0.905), SC (0.893) and in the pooled sample (0.930) while the lineage NW shows Hd lower value (0.644).

The combined (*CYTB* + *CR*) MJN network of Italian samples ([Fig. 2b](#)) shows that four mitochondrial haplogroups are present in Italy. One is formed by the TAA haplotypes of *A. amphibius*. The second is a northeastern haplogroup (corresponding to the NE clade) that occupies an intermediate position being equidistant between *A. amphibius* (32 steps) and *A. italicus* (31 steps). Within *A. italicus*, two distinct haplogroups, corresponding to the south central (SC lineage) and northwestern (NW lineage) haplotypes, are clearly visible (14 and 10 steps, respectively).

### 3.2. Population genetics

All loci are in Hardy–Weinberg equilibrium when tests were carried out within each population separately (TAA-EU, NW, SC, and NE), with few exceptions. However, when all populations are analysed together, the calculated p-values were always less than 0.05 and, therefore, the frequencies deviated from the expected Hardy–Weinberg equilibrium. No substantial differences were found in observed and expected heterozygosity values for each locus, either between subpopulations or for the total population. The estimated diversity indices for the four mitochondrial lineages show polymorphic loci in all populations, with a total number of alleles per locus ranging from a maximum of 11 to a minimum of 8. The population with the greatest number of private alleles is the NE population, while the mean allele richness is highest in the SC population of *A. italicus* ([Table A.2](#) in Appendix). None of the loci showed significant Linkage Disequilibrium (LD) across all populations, thus rejecting the hypothesis of physical association between loci on the same chromosome. The sPCA ([Fig. 3a](#)) shows a clear separation among all the populations, but as expected, the most distinct appear the one belonging to *A. amphibius* (TAA). Among the remaining Italian populations, the most distinct is *A. italicus* from northwestern Italy (NW). An individual (VEN) belonging to the NE clade falls close to the south-central Italy group. The observed sPCA pattern reflects the levels of admixture among individuals, represented by bar plots in [Fig. 3c](#). The Cross Validation (CV) score ([Fig. 3b](#)) decreases up to  $K = 7$ . However, the  $K$  decrement is steeply from  $K = 1$  to  $K = 3$  whereas, for  $K > 4$ , the CV score continues to decrease but with very small deltas. Therefore, we present the genetic structure from  $k = 2$  to  $k = 4$  as the number of putative genotypes that better describe the observed genetic structure ([Fig. 3c](#)). At  $K = 2$ , the admixture analysis ([Fig. 3c](#)) evidence two distinct clusters corresponding one to *A. amphibius* (TAA) and a second, genetically homogeneous, including all the other Italian specimens. At  $K = 3$ ,

we observe a substructure within *A. italicus* with a further distinction between populations from north-west (Piedmont, NW lineage) and all the other Italian specimens NE + SC lineages. The individuals of SC lineage and the specimen from Veneto region (VEN, part of the NE mitochondrial lineage) show a high level of admixture, whereas FVG specimens group remained homogeneous. At  $K = 4$ , we can discern further substructure among individuals of the SC lineage, with the VEN specimen and the two specimens from Calabria region showing high genetic affinity.

## 4. Discussion

The water vole of the genus *Arvicola* are known for their complex taxonomy and ecology, partly because of the presence of two distinct ecotypes. Our study focuses on water voles from Italian peninsula, an area that has been relatively understudied for this species. This area represents, also, one of the main Pleistocene species refugium of the Mediterranean area. Till now, it is believed that only one species of *Arvicola* is present, *A. italicus*, whose distribution span from the south of the peninsula to the mountain range of the Alps, which hindered a further northerly dispersal of the species during Pleistocene warm period ([Bilton et al., 1998](#)).

Our results indicate that the diversity of *Arvicola* in Italy is higher than previously described. While *A. italicus* is widespread across the entire peninsula, we detected mtDNA haplotypes in northeastern Italy (TAA, [Figs. 1 and 2](#)) that cluster with *A. amphibius*. Specifically, these haplotypes belong to the EU clade ([Brace et al., 2016; Chevret et al., 2020](#)) ([Fig. 2](#)). The genetic distinction respect to *A. italicus* is also confirmed by microsatellites, in which, the TAA population is always represented as a distinct and genetically homogeneous group ([Fig. 3a](#)). The EU clade is today distributed in the eastern part of the paleartic area, and it is thought to have spread from central European glacial refugia during early Holocene at the end of LGM, replacing the WE clade in central Europe ([Brace et al., 2016](#)). The arrival of *A. amphibius* in northeast Italy can be, therefore, dated back to the Holocene and would represent an expansion of the species from central European refugium after the last glaciation (12000bp-4500bp).

Our analysis of mitochondrial genes identified in Italy an additional and distinctive strongly supported lineage (NE, p.p. = 1/b. = 100), comprising only individuals from the northeast. This lineage exhibits significant genetic divergence respect to both *A. italicus* (SC + NW lineages; 4.4 %) and *A. amphibius* (3.7 %) ([Table 3](#)). These values lie on the threshold between intraspecific and interspecific divergence in rodents for *CYTB* ([Amori et al., 2009; Barbosa et al., 2013; Köhli et al., 2014; Michaux et al., 2002; Paupério et al., 2012; Vallejo and González-Cózar, 2012](#)).

The phylogenetic position of NE requires some considerations on the monophyly of *A. italicus* if we consider this population as belonging to this species. In fact, we observed some incongruence among analyses and markers that worth to be discussed. The intraspecific phylogeny of the species is not well resolved by *CYTB* since the NE lineage cluster with *A. amphibius* rather than with *A. italicus* ([Fig. 2a](#)), making *A. italicus* paraphyletic. However, the moderate posterior probability (p.p. = 0.7/b. = 86) suggests the possibility that the paraphyly of *A. italicus* is an artifact of the phylogenetic reconstruction based on only one mitochondrial gene. More plausible could be the hypothesis that the NE lineage could have retained ancestral haplotypes that survived in this area after the speciation from *A. amphibius*. This hypothesis is supported by the network analysis ([Fig. 2b](#)) where the NE haplogroup results equidistant from both *A. amphibius* and *A. italicus*. Furthermore, the sPCA based on nuclear microsatellite places the NE lineage in a portion of space occupied by the SC lineage ([Fig. 3a](#)). The admixture test shows, for  $K = 3$ , a sub structuring within *A. italicus*, with a separation of NW lineage and a clustering of NE with SC specimens ([Fig. 3c](#)). Signature of introgression between SC and NE is evidenced by the presence of the admixed individual from Veneto, suggesting a genetic affinity of the NE

clade with the SC lineage. To date, we cannot exclude other hypothesis, such as past hybridization between *A. amphibius* and *A. italicus*, that worth to be investigate in further studies. Indeed, is worth noting that in the Alpine area there are contact zones for several sister taxa of small mammals (Bolfková et al., 2017; Sutter et al., 2013; in reptiles also: Bagnoli et al., 2014) and the presence of gene introgression between adjacent species in small mammals is quite common (e.g. *Myodes* vole; Boratyński et al., 2011). Less common is the asymmetric gene flow that, in other vole species, has been hypothesized to be linked to diverse factors such as the differences in dispersal capability between the two sexes (Beysard et al., 2012) or to mate choice based on body size and aggressiveness (Patton and Smith, 1993; Rychlik and Zvolak, 2006; Shurtliff et al., 2013).

For what concern the intraspecific genetic diversity of *A. italicus*, we can give an additional insight respect to previous reports (Castiglia et al., 2016). Phylogenetic analysis highlights the presence of two separate and well supported lineages - NW and SC (Fig. 2a). These two lineages are also distinguishable in the network (Fig. 2b). The admixture analysis for  $K = 3$  divide *A. italicus* in two groups corresponding to NW lineage and a group including NE + SC mitochondrial lineages (Fig. 3c) suggesting firstly that NW lineage is the most differentiated and secondly, that a migration from south to northeast, despite limited, is possible.

*A. italicus* displays high intrapopulation genetic diversity, both mitochondrial and nuclear, in the south-central populations (SC lineage) and much lower variability in the northern populations (NW lineage and FVG samples, Figs. 2 and 3). This is congruent with a higher stability of *A. italicus* in the southern part of its range and is also congruent with the role of Italy as glacial refugia and the phenomenon known as “refugia within refugia” (Gómez and Lunt, 2007). The occurrence of an individual (VEN) which present shared alleles with the SC mitochondrial clade of *A. italicus* but is included in NE mtDNA lineage (Figs. 2, 3c and 3d) could suggest that recently the southern lineage expanded through north and admixed with northern populations. Both the NW and NE populations came mostly from isolated valleys (with the exception of two individuals from lowland orchard in San Genuario, see Table 1) and are both characterized by the presence of a homogeneous and differentiated nuclear genotype (Fig. 3c,  $K = 4$ ), showing low genetic variability. Traces of both the genotypes are found within SC population suggesting that the northern populations remained isolated in the past and likely underwent genetic erosion during unfavourable periods, fixing some alleles. Conversely, the southern population, who likely live in favourable condition also during LGM, retained the whole allelic diversity of the species. This is also supported by mtDNA diversity indexes (Table 4) that suggest stability in the past (high  $\pi$  and Hd values) for SC populations, contrasting with the low  $\pi$  values of northern populations NE and NW. *A. italicus*, therefore, displays a typical pattern observed in numerous small mammals in Italy, where a decrease in genetic diversity is observed as the distance from the southern refuge increases (Hewitt, 2000).

The Italian water vole *A. italicus* has been only recently separated from *A. amphibius* and, therefore, its conservation status is uncertain. However, it is believed that populations are in decline due to habitat loss as for the congeneric species (Dean et al., 2016; Jeffries et al., 1989; Bertolino et al., 2023). Moreover, there are indication of a strong predation pressure in Italy from non-native species (Bertolino et al., 2023; Loy et al., 2019; Mori and Mazza, 2019). Our results suggest uneven genetic variability across the species' range, with higher diversity in the south. And the presence of divergent mtdna lineages. Therefore, in the context of safeguarding this endemic small mammal became crucial the habitat conservation across the entire species range to preserve its genetic diversity. Future investigations could focus on expanding sampling across the entire Italian peninsula, especially in the central-northern

region (Po Valley), to enhance our understanding of the intraspecific diversity and conservation status of the species. Additionally, it would also be interesting to determine the extent of *A. amphibius* distribution in Italy and investigate whether a zone of hybridization exists between the two species.

#### CRediT authorship contribution statement

**Emanuela Solano:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Riccardo Castiglia:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Funding acquisition, Data curation, Conceptualization. **Giovanni Amori:** Writing – review & editing, Resources, Data curation, Conceptualization. **Giulia Gentile:** Writing – review & editing, Writing – original draft, Software, Investigation, Data curation. **Sandro Bertolino:** Writing – review & editing, Validation, Resources. **Dario Capizzi:** Writing – review & editing, Validation, Resources. **Petra Kranebitter:** Writing – review & editing, Resources, Funding acquisition. **Eva Ladurner:** Resources, Funding acquisition. **Luca Lapini:** Writing – review & editing, Resources, Conceptualization. **Paolo Colangelo:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

The sequences were submitted to GenBank and the accession numbers have been included in the text.

#### Acknowledgement

PC, EL, and PK were financed by the Research fund of the Museums of South Tyrol, within the project ‘File Small Mammals – the unsolved cases of the South Tyrolean small mammal fauna’, CUP H86C18000110005. RC, ES and PC were funded by the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU; Project code CN\_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP H43C22000530001 and CUP B83C22002930006 Project title “National Biodiversity Future Center - NBFC”.

#### Appendix A Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcz.2024.09.003>.

#### Appendix

**Table A.1**  
Accession numbers of the new sequenced specimens

SPECIMENS	CODE	PROVINCE	LOCALITY	Mt lineage	Accession N° <i>CYTB</i>	Accession N° <i>CR</i>
BZ01	TAA	Bolzano	Brunico	EU	PQ429245	PQ429266
BZ02		Bolzano	Brunico	EU	PQ429244	PQ429267
BZ03		Bolzano	Brunico	EU	PQ429252	-
BZ04		Bolzano	Brunico	EU	PQ429253	-
BZ05		Bolzano	Brunico	EU	PQ429246	PQ429268
BZ06		Bolzano	Brunico	EU	PQ429247	PQ429269
BZ07		Bolzano	Brunico	EU	PQ429248	PQ429270
BZ08		Bolzano	Brunico	EU	PQ429249	PQ429271
BZ10		Bolzano	Bolzano	EU	PQ429250	-
BZ11		Bolzano	Bolzano	EU	PQ429254	PQ429272
BZ12		Bolzano	Bolzano	EU	PQ429251	PQ429273
TA00	FVG	Udine	Tarvisio	NE	PQ429257	PQ429262
TA01		Carinzia	Oberthoerl	NE	PQ429260	-
TA02		Udine	Tarvisio	NE	PQ429255	PQ429263
TA03		Udine	Tarvisio	NE	PQ429259	-
TA04		Udine	Tarvisio	NE	PQ429256	PQ429264
TA05		Udine	Tarvisio	NE	PQ429258	-
TA07		Udine	Rigolato	NE	-	PQ429265
TA08		Udine	Tarvisio	NE	-	-
VEN	VEN	Belluno	Belluno	NE	PQ429261	-
PIEL 1	PIE	Cuneo	Verzuolo	NW	<a href="#">Castiglia et al. (2016)</a>	PQ429274
PIEL 2		Cuneo	Verzuolo	NW	<a href="#">Castiglia et al. (2016)</a>	PQ429275
PIEL 3		Cuneo	Verzuolo	NW	<a href="#">Castiglia et al. (2016)</a>	PQ429276
PIEL 4		Cuneo	Verzuolo	NW	<a href="#">Castiglia et al. (2016)</a>	-
PIEL 5		Cuneo	Verzuolo	NW	<a href="#">Castiglia et al. (2016)</a>	PQ429277
PIEL 6		Cuneo	Verzuolo	NW	<a href="#">Castiglia et al. (2016)</a>	PQ429278
PIEL 7		Cuneo	Verzuolo	NW	<a href="#">Castiglia et al. (2016)</a>	PQ429279
PIEL 8		Cuneo	Verzuolo	NW	<a href="#">Castiglia et al. (2016)</a>	PQ429280
A-PIEL	PIE	Vercelli	San Genuario	NW	<a href="#">Castiglia et al. (2016)</a>	PQ429281
B-PIEL		Vercelli	San Genuario	NW	<a href="#">Castiglia et al. (2016)</a>	PQ429282
POP A	POP	Pescara	Popoli	SC	<a href="#">Castiglia et al. (2016)</a>	-
POP B		Pescara	Popoli	SC	<a href="#">Castiglia et al. (2016)</a>	-
FON 1	FON	Latina	Fondi	SC	<a href="#">Castiglia et al. (2016)</a>	PQ429283
FON 2		Latina	Fondi	SC	<a href="#">Castiglia et al. (2016)</a>	PQ429284
FON 3		Latina	Fondi	SC	<a href="#">Castiglia et al. (2016)</a>	PQ429285
FON 4		Latina	Fondi	SC	<a href="#">Castiglia et al. (2016)</a>	PQ429286
CAL1	CAL	Cosenza	Sila Grande M.ts	SC	<a href="#">Castiglia et al. (2016)</a>	-
CAL2		Cosenza	Sila Grande M.ts	SC	<a href="#">Castiglia et al. (2016)</a>	-



Table A.2

Microsatellite diversity indices: (An) alleles number, (Ap) private alleles, (Ar) allelic richness, (Ho) Observed heterozygosity, (He) Expected heterozygosity, (HWp) Hardy Weinberg p-value exact.

Total population											
TAA-EU						NE					
Locus	An	Ap	Ar	Ho/He	HWp	Locus	An	Ap	Ar	Ho/He	HWp
Total population											
Ho						He					
0.70						0.84					
0.63						0.87					
0.48						0.86					
0.74						0.82					
0.48						0.85					
0.63						0.84					
0.74						0.81					
0.63						0.84					
TAA-EU						NE					
Locus	An	Ap	Ar	Ho/He	HWp	Locus	An	Ap	Ar	Ho/He	HWp
AV10	4	2	3.349802	0.86/0.68	0.922	AV10	2	0	2.844214	0.56/0.62	0.006
AV9	4	3	2.979664	0.86/0.61	0.435	AV9	6	2	3.796616	0.78/0.7	0.011
AV3	3	0	2.855622	0.71/0.62	0.764	AV3	4	2	3.026656	0.44/0.62	0.070
AV15	3	0	2.855622	1/0.62	0.148	AV15	7	1	4.555732	0.89/0.8	0.923
AV11	4	1	2.954020	0.71/0.58	0.107	AV11	6	4	4.531970	0.44/0.79	0.005
AV12	3	2	2.931313	0.71/0.66	0.360	AV12	4	1	3.354829	0.67/0.66	0.512
AV8	5	2	3.713409	1/0.7	0.786	AV8	5	1	3.006565	0.56/0.52	0.716
AV14	2	1	1.763370	0.29/0.24	1.000	AV14	4	4	3.600655	0.67/0.67	0.187
NW						SC					
Locus	An	Ap	Ar	Ho/He	HWp	Locus	An	Ap	Ar	Ho/He	HWp
AV10	5	0	4.715447	0.57/0.82	0.140	AV10	5	1	4.258042	1/0.75	0.670
AV9	3	2	2.954020	0.29/0.58	0.037	AV9	4	1	3.494794	0.5/0.66	0.343
AV3	3	2	3.553464	0.43/0.7	0.001	AV3	2	1	2.954545	0.25/0.66	0.114
AV15	3	1	2.758079	0.29/0.46	0.171	AV15	5	0	4.258042	0.75/0.75	0.649
AV11	2	1	1.497354	0.14/0.13	1.000	AV11	5	2	4.258042	0.75/0.75	0.664
AV12	5	0	3.219229	0.43/0.64	0.162	AV12	4	1	3.494794	0.75/0.66	1.000
AV8	4	0	3.402217	0.86/0.7	0.725	AV8	3	2	3.686247	0.5/0.72	0.321
AV14	4	2	3.290623	0.86/0.65	1.000	AV14	5	2	4.066589	0.75/0.69	0.785

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