

## Supplementary Material for

### Phylogeography of *Sarmarutilus rubilio* (Cypriniformes: Leuciscidae): complex genetic structure, clues to a new cryptic species and further insights into roaches phylogeny

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**Table S1.** PCR conditions and primers for each amplified marker and PCR product length. Total volume reaction = 20 µl: 2 µl of 10x NH<sub>4</sub> Buffer, 0.6 µl of 50 mM MgCl<sub>2</sub>, 0.4 µl of dNTP mix (2.5 µM each dNTP), 0.2 µl of 100 µM of each primer, 0.14 µl of 5 Uµl<sup>-1</sup> of BIOTAQ™ DNA Polymerase (Bioline) and 10–100 ng of template DNA.

Marker	Primer sequence	Reference	PCR conditions	Gene length (bp)
COI	FISHF1: 5'TCAACCAACCACAAAGACATTGGCAC3' FishR2 5'-ACTTCAGGGTGACCGAAGAATCAGAA-3'	[121]	95°C, 2 min - 30 cycles: 94°C, 30 s; 54°C, 30 s; 72°C, 1 min - 72°C, 10 min.	624
CR	ESTFOR: 5'CATCGGTCTTGTAATCCGAAGAT3' PHE1R: 5'ACATCTTCAGTGTTACGCTT3'	[122]	95°C, 2 min - 30 cycles: 94°C, 30 s; 54°C, 30 s; 72°C, 1 min - 72°C, 10 min.	929-930
Cyfun P	Cyp_un FLP1: 5'AAGTGGTGCATCGTGTGTG3' Cyp_unFL1R: 5'CAGCCTGAACAATCAAAACAG3'	[42]	94°C, 3 min - 35 cycles: 94°C, 30 s; 55°C, 30 s; 72°C, 1 min - 72°C, 10 min.	156-218*
S7	S7RPEX1F: 5'TGGCCTCTTCCTTGGCCGTC3' S7RPEX2R: 5'AACTCGTCTGGCTTTTCGCC3'	[123]	95°C, 3 min - 30 cycles: 95°C, 30 s; 54°C, 1 min; 72°C, 2 min - 72°C, 10 min.	319-861*

\*total alignment including gaps

## References

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**Table S2.** COI Sequences retrieved from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and Barcode Of Life Data SYSTEM ([www.boldsystems.org](http://www.boldsystems.org), in yellow) included in the phylogenetic reconstruction.

Species label from databases (Genus <i>Rutilus</i> )	Accession Number	Current valid species name (Eschmeyer Catalogue of Fishes, <a href="https://www.calacademy.org/scientists/projects/eschmeyers-catalog-of-fishes">https://www.calacademy.org/scientists/projects/eschmeyers-catalog-of-fishes</a> )	Country (basin)	ID site (only <i>S. rubilio</i> , see Figure 1)
<i>R. albus</i>	MG806869	<i>Leucos albus</i>	Albania (Drin)	
<i>R. albus</i>	KJ554332	<i>Leucos albus</i>	Albania (Skadar lake)	
<i>R. albus</i>	KJ554244	<i>Leucos albus</i>	Albania (Skadar lake)	
<i>R. albus</i>	KJ554242	<i>Leucos albus</i>	Albania (Skadar lake)	
<i>R. aula</i>	KJ554532	<i>Leucos aula</i>	Italy (Po)	
<i>R. aula</i>	KJ554171	<i>Leucos aula</i>	Croatia (Zadar)	
<i>R. aula</i>	HM560323	<i>Leucos aula</i>	Croatia (Bacisca lake)	
<i>R. aula</i>	MG806870	<i>Leucos aula</i>	Croatia (Zrmanja)	
<i>R. basak</i>	MG806871	<i>Leucos basak</i>	Bosnia and Herzegovina (Neretva)	
<i>R. basak</i>	HM560325	<i>Leucos basak</i>	Bosnia and Herzegovina (Neretva)	
<i>R. basak</i>	KJ554498	<i>Leucos basak</i>	Bosnia and Herzegovina (Neretva)	
<i>R. basak</i>	KJ554191	<i>Leucos basak</i>	Bosnia and Herzegovina (Mandek)	
<i>R. frisii</i>	MG806872	<i>Rutilus frisii</i>	Bulgaria (Rezowska)	
<i>R. frisii</i>	KJ554245	<i>Rutilus frisii</i>	Bulgaria (Rezowska)	
<i>R. frisii</i>	MT756353	<i>Rutilus frisii</i>	Iran (Gorgan Gulf Caspian Sea)	
<i>R. frisii</i>	MT756352	<i>Rutilus frisii</i>	Iran (Gorgan Gulf Caspian Sea)	
<i>R. heckelii</i>	KJ554478	<i>Rutilus lacustris</i>	Greece (Volvi lake)	
<i>R. heckelii</i>	KJ554441	<i>Rutilus lacustris</i>	Greece (Volvi lake)	
<i>R. heckelii</i>	KJ554358	<i>Rutilus lacustris</i>	Greece (Volvi lake)	
<i>R. heckelii</i>	KJ554261	<i>Rutilus lacustris</i>	Greece (Volvi lake)	
<i>R. kutum</i>	AQM026-16	<i>Rutilus kutum</i>	Iran (Mazandaran Province)	
<i>R. kutum</i>	AQM027-16	<i>Rutilus kutum</i>	Iran (Mazandaran Province)	
<i>R. kutum</i>	AQM028-16	<i>Rutilus kutum</i>	Iran (Mazandaran Province)	

<i>R. kutum</i>	AQM029-16	<i>Rutilus kutum</i>	Iran (Mazandaran Province)	
<i>R. lacustris</i>	MT756380	<i>Rutilus lacustris</i>	Iran (Gorgan Gulf Caspian Sea)	
<i>R. lacustris</i>	HQ561910	<i>Rutilus lacustris</i>	Georgia (Rioni)	
<i>R. lacustris</i>	HQ561909	<i>Rutilus lacustris</i>	Georgia (Rioni)	
<i>R. lacustris</i>	MG806873	<i>Rutilus lacustris</i>	Greece (Struma)	
<i>R. meidingeri</i>	KR477255	<i>Rutilus meidingeri</i>	Austria (Mondsee lake)	
<i>R. meidingeri</i>	KR477254	<i>Rutilus meidingeri</i>	Austria (Mondsee lake)	
<i>R. meidingeri</i>	KR477253	<i>Rutilus meidingeri</i>	Austria (Mondsee lake)	
<i>R. meidingeri</i>	KR477099	<i>Rutilus meidingeri</i>	Austria (Mondsee lake)	
<i>R. ohridanus</i>	MG806874	<i>Leucos basak</i>	Albania (Ohrid lake)	
<i>R. ohridanus</i>	KJ554509	<i>Leucos basak</i>	Albania (Ohrid lake)	
<i>R. ohridanus</i>	KJ554414	<i>Leucos basak</i>	Albania (Ohrid lake)	
<i>R. ohridanus</i>	KJ554350	<i>Leucos basak</i>	Albania (Ohrid lake)	
<i>R. panosi</i>	MG806875	<i>Leucos panosi</i>	Greece (Trichonis lake)	
<i>R. panosi</i>	KJ554528	<i>Leucos panosi</i>	Greece (Trichonis lake)	
<i>R. panosi</i>	KJ554495	<i>Leucos panosi</i>	Greece (Trichonis lake)	
<i>R. panosi</i>	KJ554396	<i>Leucos panosi</i>	Greece (Trichonis lake)	
<i>R. pigus</i>	HM560327	<i>Rutilus pigus</i>	Italy (Adda)	
<i>R. pigus</i>	HM560326	<i>Rutilus pigus</i>	Italy (Adda)	
<i>R. pigus</i>	KJ554485	<i>Rutilus pigus</i>	Italy (Como lake)	
<i>R. pigus</i>	KJ554328	<i>Rutilus pigus</i>	Italy (Como lake)	
<i>R. prespensis</i>	KJ554482	<i>Leucos basak</i>	Albania (Prespa lake)	
<i>R. prespensis</i>	KJ554471	<i>Leucos basak</i>	Albania (Prespa lake)	
<i>R. prespensis</i>	KJ554468	<i>Leucos basak</i>	Albania (Prespa lake)	
<i>R. prespensis</i>	KJ554445	<i>Leucos basak</i>	Albania (Prespa lake)	
<i>R. rubilio</i>	KJ554475	<i>Sarmarutilus rubilio</i>	Italy (Arno)	ARN-Cas
<i>R. rubilio</i>	KJ554114	<i>Sarmarutilus rubilio</i>	Italy (Arno)	ARN-Cas
<i>R. rubilio</i>	KJ554324	<i>Sarmarutilus rubilio</i>	Italy (Arno)	ARN-Ter
<i>R. rubilio</i>	KJ554212	<i>Sarmarutilus rubilio</i>	Italy (Arno)	ARN-Ter
<i>R. rubilio</i>	KJ554352	<i>Sarmarutilus rubilio</i>	Italy (Tiber)	TIB-Ang

<i>R. rubilio</i>	KJ554250	<i>Sarmarutilus rubilio</i>	Italy (Tiber)	TIB-Ang
<i>R. rubilio</i>	KJ554361	<i>Sarmarutilus rubilio</i>	Italy (Tiber)	TIB-Mon
<i>R. rubilio</i>	KJ554088	<i>Sarmarutilus rubilio</i>	Italy (Tiber)	TIB-Mon
<i>R. rubilio</i>	KJ554380	<i>Sarmarutilus rubilio</i>	Italy (Sisto)	SIS
<i>R. rubilio</i>	KJ554226	<i>Sarmarutilus rubilio</i>	Italy (Sisto)	SIS
<i>R. rubilio</i>	KJ554435	<i>Sarmarutilus rubilio</i>	Italy (Fondi lake)	FON
<i>R. rubilio</i>	KJ554290	<i>Sarmarutilus rubilio</i>	Italy (Fondi lake)	FON
<i>R. rubilio</i>	KJ554508	<i>Sarmarutilus rubilio</i>	Italy (Volturno)	VOL
<i>R. rubilio</i>	KJ554364	<i>Sarmarutilus rubilio</i>	Italy (Volturno)	VOL
<i>R. rubilio</i>	KJ554200	<i>Sarmarutilus rubilio</i>	Italy (Volturno)	VOL
<i>R. rubilio</i>	KJ554118	<i>Sarmarutilus rubilio</i>	Italy (Volturno)	VOL
<i>R. rubilio</i>	KJ554409	<i>Sarmarutilus rubilio</i>	Italy (Crati)	CRA
<i>R. rubilio</i>	KJ554274	<i>Sarmarutilus rubilio</i>	Italy (Crati)	CRA
<i>R. rubilio</i>	KJ554129	<i>Sarmarutilus rubilio</i>	Italy (Crati)	CRA
<i>R. rutilus</i>	KT989765	<i>Rutilus rutilus</i>	Russia (Plescheevo lake)	
<i>R. rutilus</i>	HQ961042	<i>Rutilus rutilus</i>	Czech Republic (Ohre)	
<i>R. rutilus</i>	MW473258	<i>Rutilus rutilus</i>	Germany (Rhine)	
<i>R. rutilus</i>	HM392103	<i>Rutilus rutilus</i>	Germany (Danube)	
<i>R. virgo</i>	HM392106	<i>Rutilus virgo</i>	Germany (Danube)	
<i>R. virgo</i>	HM392104	<i>Rutilus virgo</i>	Germany (Vils)	
<i>R. virgo</i>	HM392102	<i>Rutilus virgo</i>	Germany (Danube)	
<i>R. virgo</i>	MG806878	<i>Rutilus virgo</i>	Croatia (Sava)	
<i>R. ylikiensis</i>	MG806879	<i>Leucos ylikiensis</i>	Greece (Yliki lake)	
<i>R. ylikiensis</i>	KJ554524	<i>Leucos ylikiensis</i>	Greece (Kifissos)	
<i>R. ylikiensis</i>	KJ554516	<i>Leucos ylikiensis</i>	Greece (Kifissos)	
<i>R. ylikiensis</i>	KJ554316	<i>Leucos ylikiensis</i>	Greece (Kifissos)	

**Table S3.** COI (a) and CR (b) diagnostic sites identified among the most frequent sequences of the three haplogroups found in Italian specimens. Deletions are marked with “-”.

(a) Diagnostic COI (624 bp) sites

			(a) Diagnostic COI (521 bp) sites																											
Position/ Haplotype			1	1	2	2	2	2	2	2	2	2	2	3	3	3	3	4	4	4	4	4	4	4	4	5	5	5	5	6
	5	9	4	9	1	1	5	5	5	7	7	7	8	9	0	0	3	8	0	2	3	5	6	7	9	0	2	3	5	1
	4	6	4	8	6	9	0	2	6	0	6	9	5	4	3	9	0	4	8	9	5	3	2	7	5	4	2	4	5	2
HpA01	G	T	C	G	T	G	T	G	C	C	A	T	C	A	A	G	C	G	C	T	G	A	G	A	C	C	A	A	T	C
HpB01	G	T	C	G	T	G	T	G	C	C	G	T	C	A	A	A	C	G	T	C	G	G	G	A	C	T	G	A	T	T
HpC01	A	C	T	A	C	A	C	A	T	T	A	C	T	G	G	G	T	C	T	T	A	G	A	G	A	C	G	G	C	T

(b) Diagnostic CR (930 bp) sites

c) Diagnostic CR (500 bp) sites																																			
Position/ Haplotype	1 3 3 3 3 4 4 4 4 4 5 5 5 6 6 6 6 7 7 7 8 8 8 8 8 8 8 8 9 9 9																																		
	2 5 8 4 7 7 7 9 1 6 6 7 9 1 5 6 0 1 5 7 2 2 8 0 3 3 4 7 7 8 9 9 1 3																																		
	7 5 5 6 2 1 2 6 3 7 3 5 3 4 8 5 7 8 0 3 0 4 5 3 8 5 9 4 3 7 9 4 7 6 0																																		
HpA01	C	A	G	T	C	C	G	G	T	G	T	T	A	T	G	A	C	T	T	T	C	A	T	A	G	G	G	A	G	T	G	G	T	A	C
HpB01	G	A	A	T	C	C	G	G	A	G	C	C	A	A	G	A	T	T	T	T	T	T	T	G	A	A	G	A	G	T	A	G	T	A	C
HpC01	C	G	A	C	T	A	T	T	A	T	T	T	G	T	A	T	T	C	C	C	T	T	A	A	A	A	A	G	A	G	A	A	-	G	T

**Table S4.** Diagnostic fragment of nuclear marker Cyfun P in Italian specimens subset. Individuals with specific VAR sequences are highlighted in green, variable sites/indels in yellow; mtDNA lineage of each specimen is also reported. Missing data are marked with N, deletions with -.

	CyfunP GenBank Accession Number	MtDNA haplogroup	1 4 6	1 4 7	1 4 8	1 4 9	1 5 0	1 5 1	1 5 2	1 5 3	1 5 4	1 5 5	1 5 6	1 5 7	1 5 8	1 5 9	1 6 0	1 6 1	1 6 2	1 6 3	1 6 4	1 6 5	1 6 6	1 6 7	1 6 8	1 6 9	1 7 0	1 7 1	1 7 2	1 7 3	1 7 4	1 7 5	1 7 6	1 7 7	1 7 8	1 7 9	1 8 0	1 8 1	1 8 2	1 8 3	1 8 4	1 8 5	1 8 6	1 8 7	1 8 8	1 8 9	1 9 0	1 9 1	1 9 2		
VAR1_46	OM966266	C	A	A	C	A	A	A	A	A	A	C	A	C	A	R	A	A	A	-	-	-	A	A	A	T	A	A	A	A	G	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
VAR1_49	OM966267	C	A	A	C	A	W	A	A	A	A	C	A	C	A	N	A	A	A	-	-	-	A	A	A	T	A	A	A	A	G	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
VAR2_02	OM966268	A	A	A	C	A	A	A	A	A	A	C	A	C	A	G	A	A	A	-	-	-	A	A	A	T	A	A	A	A	G	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
VAR2_09	OM966269	C	A	A	C	A	A	A	A	A	A	C	A	C	A	N	A	A	A	-	-	-	A	A	A	T	A	A	A	A	G	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
VAR2_11	OM966270	C	A	A	C	A	A	A	A	A	A	C	A	N	N	N	N	N	N	-	-	-	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		
VAR2_25	OM966271	C	A	A	C	A	A	A	A	A	A	C	A	C	A	N	A	A	A	-	-	-	A	A	A	T	A	A	A	A	G	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
VAR2_32	OM966272	C	A	A	C	A	W	A	A	A	A	C	A	C	A	N	A	A	A	-	-	-	A	A	A	T	A	A	A	A	G	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
VAR1_33	OM966273	A	T	A	A	A	A	A	A	A	T	A	A	A	A	N	T	A	A	A	A	T	A	A	A	T	A	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
VAR1_43	OM966274	A	T	A	A	A	W	A	A	A	T	A	A	A	A	T	A	A	A	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
VAR2_04	OM966275	C	T	A	A	A	A	A	A	A	T	A	A	A	A	T	A	A	A	A	T	A	A	A	T	A	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
VAR2_05	OM966276	C	T	A	A	A	A	A	A	A	T	A	A	A	A	T	A	A	A	A	T	A	A	A	T	A	A	A	A	G	G	A	A	G	T	T	T	T	C												
VAR2_06	OM966277	C	T	A	A	A	A	A	A	A	T	A	A	A	A	T	A	A	A	A	T	A	A	A	T	A	A	A	A	G	G	A	A	G	T	T	T	T	C												
VAR2_28	OM966278	A	T	A	A	A	A	A	A	A	T	A	A	A	A	T	A	A	A	A	T	A	A	A	T	A	A	A	A	G	G	A	A	G	T	T	T	T	C												
TIB1_26	OM966279	A	T	A	A	A	T	A	A	A	T	A	A	A	A	T	A	A	A	A	T	A	A	A	T	A	A	A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
SCR_01	OM966280	A	T	A	A	A	A	A	A	A	T	A	A	A	A	T	A	A	A	A	T	A	A	A	T	A	A	A	T	A	A	A	G	G	A	A	G	T	T	T	T	C									
SCR_02	OM966281	A	T	A	A	A	A	A	A	A	T	A	A	A	A	T	A	A	A	A	T	A	A	A	T	A	A	A	T	A	A	A	G	G	A	A	G	T	T	T	T	C									

**Table S5.** Inter- (below the diagonal) and intra-haplogroup (diagonal, in grey) genetic mean distances (CR); pairwise  $\Phi_{ST}$  between CR haplogroups (above the diagonal).

	A	B	C
A	0.0009 ( $\pm 0.0005$ )	0.94***	0.97***
B	0.0151 ( $\pm 0.0040$ )	0.0013 ( $\pm 0.0005$ )	0.96***
C	0.0325 ( $\pm 0.0062$ )	0.0291 ( $\pm 0.0058$ )	0.0005 ( $\pm 0.0005$ )



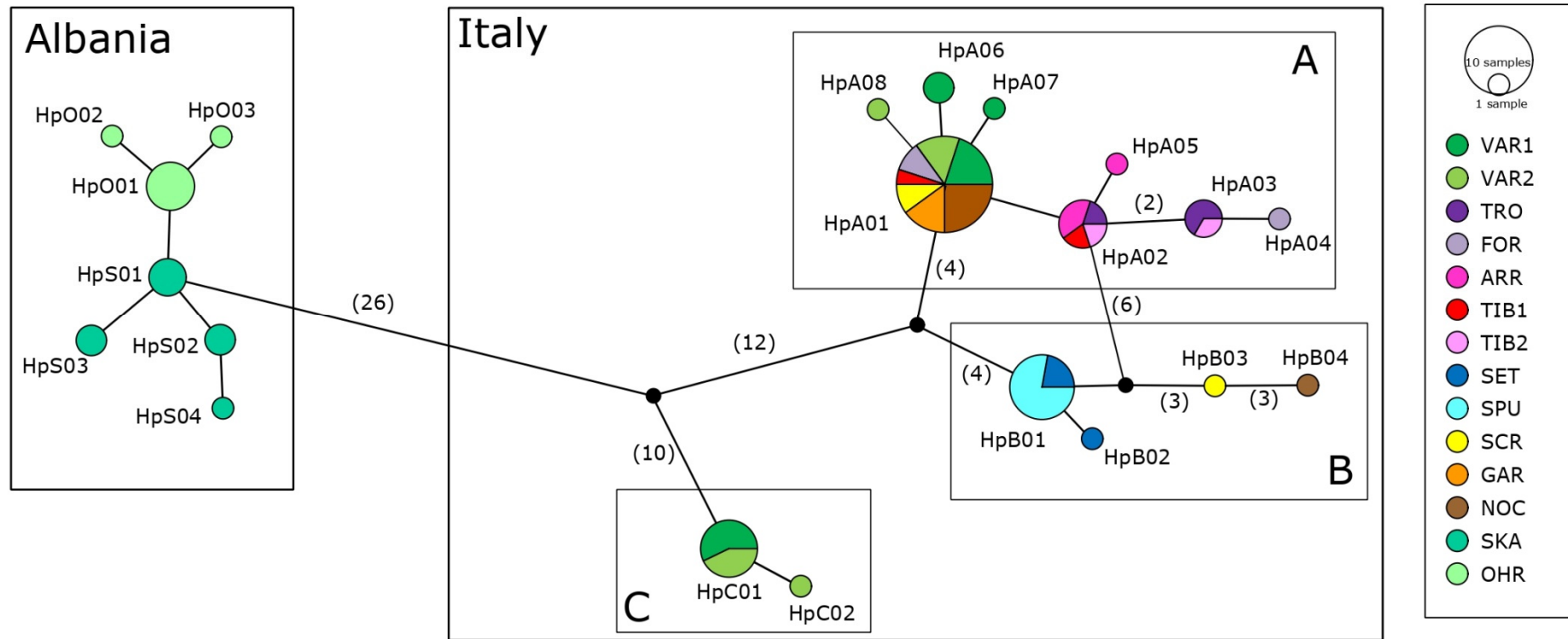
**Table S6.** Population  $\Phi_{ST}$  (CR) considering all the populations as they are (a) and splitting individuals by haplogroup for each one (b). Significance thresholds: \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ .

(a)

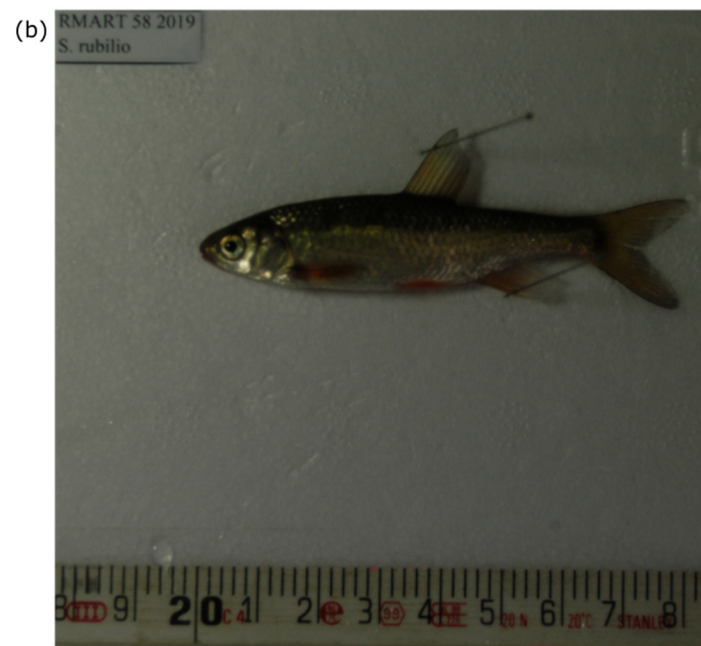
		Haplogroup A/C		Haplogroup A						Haplogroup A/B		Haplogroup B	
		VAR1	VAR2	TRO	FOR	ARR	TIB1	TIB2	GAR	SCR	NOC	SET	SPU
HpA/C	VAR1	-											
	VAR2	-0.05	-										
HpA	TRO	0.29	0.3	-									
	FOR	0.28	0.33	0.05	-								
	ARR	0.31***	0.35	0.68***	0.64***	-							
	TIB1	0.38***	0.43***	0.13	0.13	0.45***	-						
	TIB2	0.06	0.16	0.20	0.19	0.47	0.13	-					
	GAR	0.33	0.38	0.07	0.08	0.61***	0.15***	0.01	-				
HpA/B	SCR	0.32***	0.38**	0.18	0.17	0.36***	0.21***	0.01	0.11	-			
	NOC	0.09	0.16	0.42***	0.38***	0.31***	0.41***	0.01	0.36***	0.12	-		
HpB	SET	0.65***	0.58***	0.98***	0.97***	0.96***	0.95***	0.96***	0.97***	0.90***	0.87***	-	
	SPU	0.51***	0.47***	0.98***	0.96***	0.95***	0.95***	0.95	0.97***	0.88***	0.79***	0.12	-

(b)

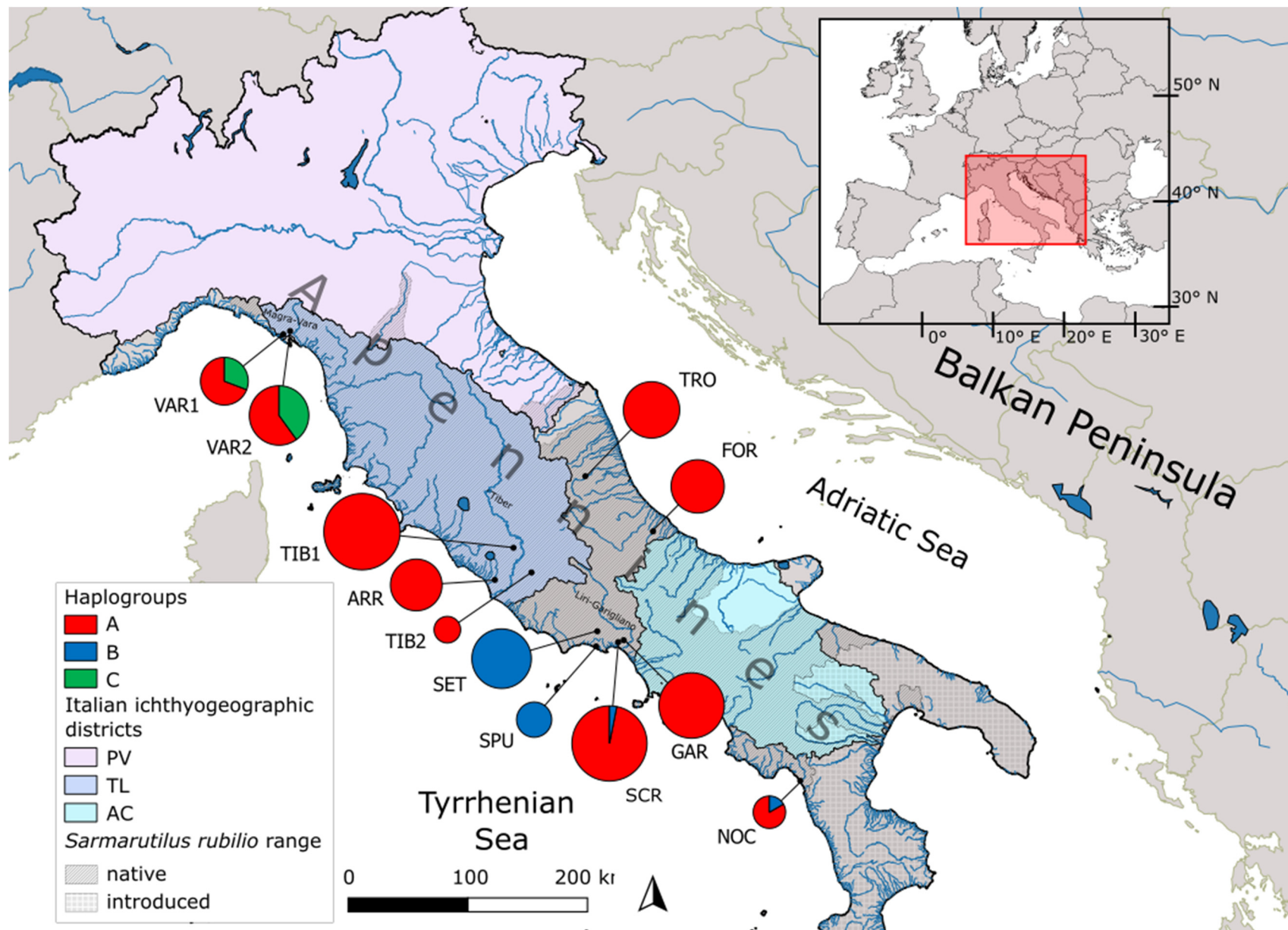
		Haplogroup C		Haplogroup A										Haplogroup B			
		VAR1_C	VAR2_C	VAR1_A	VAR2_A	TRO	FOR	ARR	TIB1	TIB2	SCR_A	GAR	NOC_A	SET	SPU	SCR_B	NOC_B
HpC	VAR1_C	-															
	VAR2_C	-0.07	-														
HpA	VAR1_A	0.99	0.99***	-													
	VAR2_A	0.99	0.99***	-0.03	-												
	TRO	0.99***	0.99***	-0.04	0.01	-											
	FOR	0.99	0.99***	0.02	0.04	0.05	-										
	ARR	0.97***	0.98***	0.63	0.63***	0.68***	0.64***	-									
	TIB1	0.97***	0.98***	0.09	0.10	0.13	0.13	0.45***	-								
	TIB2	0.97	0.98	0.22	0.18	0.20	0.19	0.47	0.13	-							
	SCR_A	0.97***	0.97***	0.23	0.19	0.28**	0.27**	0.49***	0.28***	0.09	-						
	GAR	0.99***	0.99**	0.03	0.03	0.07	0.08*	0.61***	0.15***	0.01	0.17	-					
	NOC_A	0.99	0.99***	1.00***	0.89	0.92***	0.84***	0.64***	0.60***	0.55	0.35	0.67	-				
HpB	SET	0.98***	0.98***	0.97***	0.97***	0.98***	0.97***	0.96***	0.95***	0.96***	0.94***	0.97***	0.97***	-			
	SPU	0.98	0.98***	0.98***	0.98***	0.98***	0.98***	0.95***	0.95***	0.95	0.93***	0.97***	0.98	0.12	-		
	SCR_B	0.98	0.98	1.00	0.99	0.99	0.98	0.94	0.95	0.94	0.93	0.98	1.00	0.89	0.87	-	
	NOC_B	0.98	0.98	1.00	0.99	0.99	0.98	0.94	0.95	0.94	0.93	0.98	1.00	0.92	0.91	1.00	-



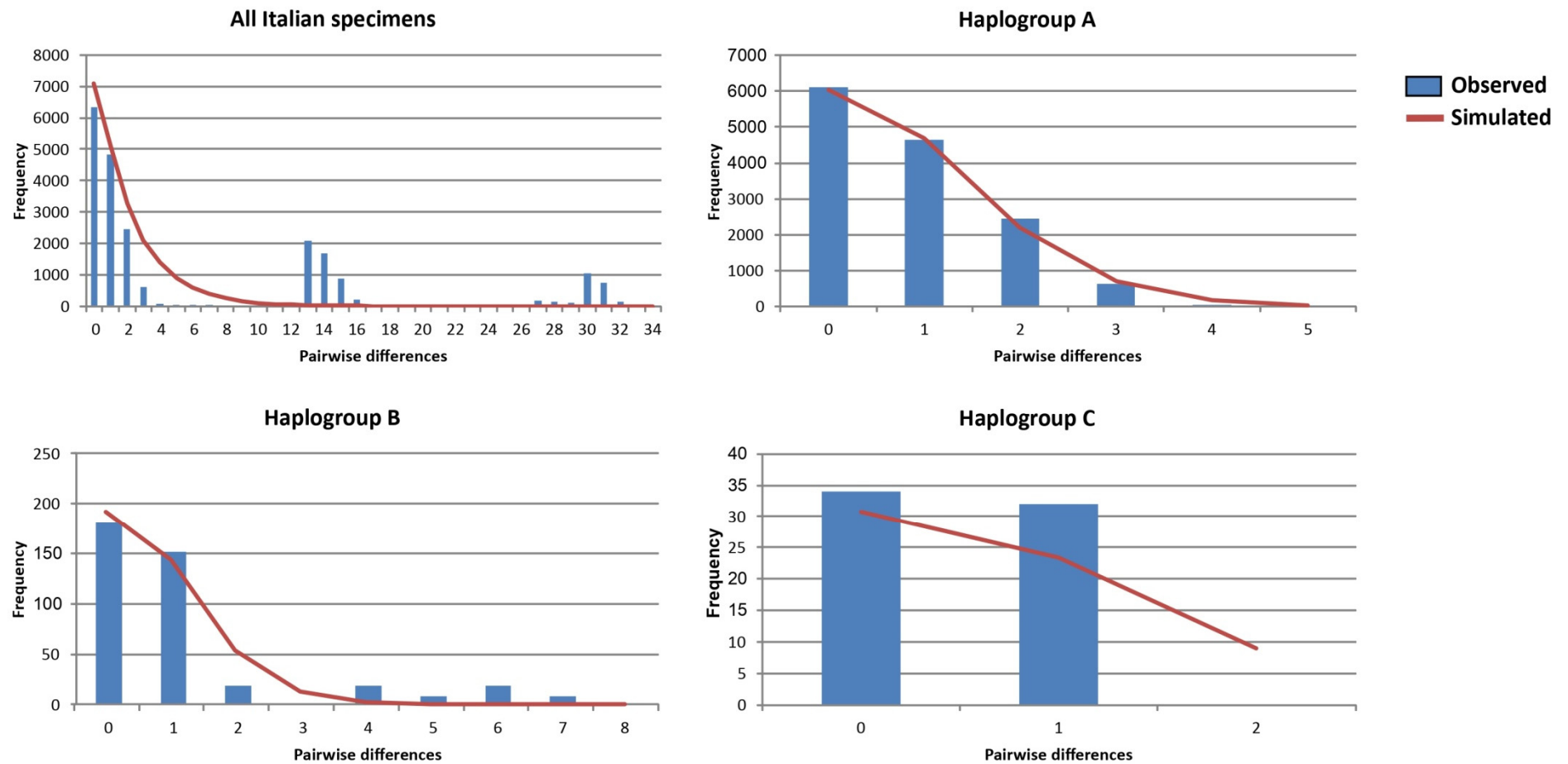
**Figure S1.** Haplotype network based on COI sequences obtained in this study. Each circle corresponds to one haplotype and its dimension is proportional to the haplotype frequency. The number of nucleotide substitutions between haplotypes is indicated in parenthesis. Population abbreviations refer to Table 1. Letters (A, B, C) and boxes refer to *S. rubilio* haplogroups as indicated in Figure 2.



**Figure S2.** Specimens with mtDNA and Cyfun P belonging to lineage C (a) and lineage A (b).



**Figure S3.** Spatial haplogroups distribution in *S. rubilio*, based on 208 Italian CR sequences.



**Figure S4.** Observed mismatch distributions (bars) and expected mismatch distributions under the sudden expansion model (solid line) of CR sequences.