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Chromosome analysis on Central and Southern Italy population of the common toad, *Bufo bufo* (Amphibia, Anura)

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Abstract

Amphibians constitute a very good model to explore the historical aspects of species distributions due to their low dispersal capacity and low individual vagility. Bufonidae are one of the most speciose family of Anura, including taxa, such as *Bufo bufo*, widespread in Eurasian regions.

We performed a karyological study with standard and sequential C-banding + fluorochromes (Chromomycin A_3 (CMA) and Diamidinophenylindole (DAPI) on several tadpoles from different populations of Central and Southern Italy. All the examined tadpoles exhibited the standard *Bufo* karyotype of 2n = 22 biarmed chromosomes, with the first six pairs larger than the other five (7 - 11) pairs and NOR associated heterochromatin distal on the long arms of the 6th chromosome pair, that was also the only chromosome CMA-positive region. C-banding evidenced centromeric heterochromatin, DAPI positive, on all the chromosomes in all the studied populations from Central Italy. The Southern Italy populations differed in additional paracentromeric C-bands on the short arms of chromosomes 1, 3 and 5. These results support the partition of Central populations of *B. bufo* from the Southern ones, as evidenced also from molecular phylogenetic studies.

Keywords: *Bufo bufo*, C-banding, heterochromatin, karyotype, NORs

Riassunto

Gli anfibi costituiscono un ottimo modello per esplorare gli aspetti storici della distribuzione delle specie a causa della loro bassa capacità di dispersione e della bassa vagilità individuale. I bufonidi sono una delle famiglie di Anuri più ricche di specie, tra cui il rospo comune, *Bufo bufo*, diffuso nelle regioni eurasiatiche.
Abbiamo eseguito uno studio cariologico con tecniche standard di bandeggio C e sequenziali con fluorocromi (Cromomicina A_3 (CMA) e Diamidinofenilindolo (DAPI) su diversi girini di diverse popolazioni del Centro e Sud Italia. Tutti i girini esaminati presentavano il cariotipo *Bufo* standard, di 2n = 22 cromosomi a due braccia, con le prime sei coppie più grandi delle altre cinque (7 - 11), e l'eterocromatina NOR-associata distale sui bracci lunghi della sesta coppia di cromosomi, che era anche l'unica regione cromosomica CMA-positiva. Il bandeggio C ha evidenziato eterocromatina centromerica, DAPI positiva, su tutti i cromosomi in tutte le popolazioni studiate dell'Italia centrale. Le popolazioni dell'Italia meridionale differivano per bande C paracentromeriche aggiuntive sui bracci corti dei cromosomi 1, 3 e 5. Questi risultati supportano la suddivisione delle popolazioni centrali di *B. bufo* da quelle meridionali, come evidenziato anche da studi filogenetici molecolari

Parole chiave: *Bufo bufo,* pattern di Bandeggio-C, eterocromatina, cariotipo, NORs

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Introduction

DNA sequencing has provided great advancement in biological studies, such as phylogenetics, systematics and taxonomy, nevertheless conventional and molecular cytogenetic analyses are still relevant to study the genomic/chromosomal changes during evolution. Comparative chromosome analyses can be useful to identify plesiomorphic and apomorphic characters and the occurrence of different evolutionary lineages (Mezzasalma et al. 2015, 2021). Chromosome rearrangements may either precede or follow molecular evolution and directly promote the speciation, or as by product after phylogenetic diversification (King 1993; Mezzasalma et al. 2017). In either case, they represent discrete evolutionary markers able to detect different evolutionary trends in the taxa studied (Olmo, 2008; Mezzasalma et al. 2022a, 2002b).
Amphibians constitute a very good model to explore the historical aspects of species distributions due to their low dispersal capacity and exhibit low individual vagility, often accompanied by high philopatry to natal sites retention (Beebee, 1996). Moreover, they are very sensitive to climatic

changes, which make them optimal organisms for discriminating the effects of glacial cycles and other environmental changes upon their genetic structure and biogeographic patterns (Zeisset and Beebee, 2008).

Bufonidae is the third largest Anura family with 640 species so far recognised (AmphibiaWeb 2022; Frost 2022), among which *Bufo bufo*, widespread in almost all Eurasian regions.

Bufo bufo is part of a species complex, formerly considered as its subspecies, namely: the Caucasian toad (*B. verrucosissimus*); the Japanese common toad (*B. japonicus*), the European common toad (*B.bufo*) and a new species, *B. eichwaldi*, described by Litvinchuk et al. (2008), morphologically and genetically distinct from *B. bufo*, living in south Azerbaijan and Iran. (Recuero et al. 2012; Arntzen et al. 2013, 2016; 2017). Garcia-Porta et al. (2012) analysed the phylogenetic relationships between the Eurasian and North African species of the *B. bufo* group and specified a first split of *B. eichwaldi* from the main lineage occurred around to thirteen - nine million years ago. Next split of *B. spinosus* dated about five million years ago. Finally, the splitting between *B. bufo* and *B. verrucosissimus* occurred about three million years ago during the Pleistocene.

Several karyological studies, using different chromosome banding, have been performed on *B. bufo* species group (Schmid 1978; Birstein and Mazin 1982; Matsui et al., 1985; Spasić-Bošković, et al. 2000; Skorinov et al. 2018; Guzmán-Markevich et al. 2022). Concerning the Italian population, the only one study was conducted by Morescalchi (1964), who used standard chromosome staining, on a population from Southern Italy.

In this paper we carried out a chromosomal study using standard and C-banding staining methods on tadpoles of *B. bufo* from several Central and Southern Italy populations aiming to detect eventual differences on the chromosomes and heterochromatin distribution among the considered populations.

Material and Methods

The number and origin of the examined tadpoles of the common toad, *B. bufo* are given in table 1.

Specimen identity of all studied tadpoles (Table 1) was determined by the molecular barcoding method, using a segment of the mitochondrial 16S rDNA gene, which is widely used in phylogenetic analyses on this toad taxon (e.g. Kutrup et al. 2006; Garcia-Porta et al. 2012; Recuero et al. 2012; Arntzen et al, 2017; Chiocchio et al., 2021). DNA was extracted from cell suspensions following Sambrook et al. (1989). Primer pairs were: 16Sa (CGCCTGTTTATCAAAAACAT) and 16Sb (CCGGTCTGAAACTCAGATCAGT) (Palumbi et al. 1991), allowing to amplify a segment of about 550 bp. The PCR running parameters were: 5 min. at 95◦C (denaturation step); 35 cycles at 94◦C for 30 s; 55◦C for 30 s and 72◦C for 1 min. (amplification cycles); 7 min. at 72◦C (termination step). After gel electrophoresis on 1.5% agarose gel, the bands of the amplified products were excised, purified from the gel with GenElute kit (Sigma), and sequenced in both orientations using the BigDye Terminator kit v1.1 and the automatic sequencer ABI Prism 310 (Applied Biosystems, Foster City). Chromatograms were checked and edited using Chromas Lite 2.6.6 and BioEdit 7.2.6.1

Table 1: Number and origin of studied tadpoles of *B. bufo*; cl.e6 and cl.e7 refer to the clades of the tree designed by Garcia-Porta et al. (2012).

Origin	Nr.	% Id, vs Seq- GenBank
Conero, Ancona (Marche)	2	100% vs JQ348788 (Monteleone d'Orvieto, PG) (cl. e6)
Minturno, Latina (Lazio)	2	100% vs JQ348786 (Campa di Segni, Roma) (cl. e6)
Rio Santa Croce, Formia, Latina (Lazio)	3	100% vs JQ348786 (Campa di Segni, Roma) (cl. e6)
Lago Penitro, Formia, Latina (Lazio)	2	100% vs JQ348786 (Campa di Segni, Roma) (cl. e6)
Cesinali, Serino, Avellino (Campania)	3	100% vs AY555020 (Matera) (cl. e7)
Altamura, Bari (Puglia)	2	100% vs JQ348795 (Bari) (cl. e7)
Gallo Matese, Caserta (Campania)	2	99.6% vs AY555020 (Matera) (cl. e7)
Lago Letino, Letino, Caserta (Campania)	2	99.6% vs AY555020 (Matera) (cl. e7)
Agnone Cilento, Salerno (Campania)	2	99,8% vs JQ348794 (Piaggine, Salerno) (cl. e7)
Montecorice, Salerno (Campania)	3	99,8% vs JQ348794 (Piaggine, Salerno) (cl. e7)
San Nicola Arcella, Cosenza (Calabria)	2	100% vs JQ348763 (Cetraro, CS) (cl. e7)

(Hall 1999). Sequences were deposited in GenBank: OQ301661 - OQ301672.

Chromosome analysis

The chromosome analysis was performed using the scraping method (Sharma and Sharma 1980), as modified by Petraccioli et al. (2015). Chromosomes were derived from intestine (cleaned form debris) as described by Petraccioli et al. (2012). In brief, after immersion of the specimen in tricaine methanesulfonate (0,1%), the intestine of each tadpole was removed and incubated for two hours in one ml of calf serum and inactivated at 56°C for 30 min in a solution containing 50 µl of colcemid at 10 µg/ml. Subsequently, the intestine was incubated for 30 min. in hypotonic solution (KCl 0.075 M + sodium citrate 0.5%, 1:1) and fixed for 15 min. in methanol: acetic acid, 3:1. Cell dissociation of the intestine was made on a 100-mesh sieve and chromosomes were prepared dropping 25 µl of the obtained cell suspensions on the slides. The chromosome staining was performed with traditional staining (5% Giemsa solution at pH 7 for 10 min) and sequential C-banding + CMA_3 + DAPI according to Mezzasalma et al. (2022a). Karyotype reconstruction was performed after scoring at least five plates per sample and chromosomes were classified following Levan et al. (1964).

Results

The results of 16S analysis confirmed the taxonomic attribution of the studied tadpoles to the taxon *Bufo bufo* (see Table 1 for the results of queries in GenBank deposited). Within each population the tadpoles show an unique haplotype, but it should be stressed that the inter-populational diversification of the segment of 16S sequences here considered is very scarce, e.g. the identity is 99.81%. between the two more distant population studied, San Nicola Arcella (Cosenza) and Conero (Marche).

Chromosomes were obtained from tadpoles of all populations, except for those from Cesinali (Avellino). All the other tadpoles, regardless of the origin, showed a karyotype of 2n = 22 biarmed chromosomes, with the first six pairs (1-6) distinctively bigger than the other five pairs (7-11) (Fig. 1). Sequential C-banding + CMA + DAPI evidenced that all chromosomes were uniformly stained with the first fluorochrome, except for the peritelomeric regions of long arms of chromosomes of the sixth pair that were strongly CMA-positive (Fig. 1). C-banding + DAPI showed centromeric C-band positive to this fluorochrome on all chromosomes of tadpoles from Conero (Ancona), Minturno (Latina), Rio Santa Croce (Latina), Lago Penitro (Latina), and Lago Letino (Caserta) and Gallo Matese (Caserta) (Fig. 1). The tadpoles from Altamura (Bari); Agnone Cilento (Salerno), Montecorice (Salerno), Corleto (Salerno) and San Nicola Arcella (Cosenza) in addition to centromeric C-bands on all chromosomes showed para-centromeric C-bands DAPI positive on the short arms of the chromosomes of pairs 1, 3 and 5 (Fig. 1 D'-F').

Discussion

The first chromosomal study on common toad was conducted on French specimens and date back to the first years of the last century (Lebrun, 1902). This study reported an inexact number of elements, that successive analyses on European and Asian specimens established to be 2n = 22 (Stohler, 1927-1928; Minouchi and Iriki, 1931; Tchou-Su, 1931; Galgano, 1933; Witschi, 1933; Wickbom, 1945). Next studies on specimens from Monte Cerreto (Salerno, Italy) by Morescalchi (1964) and from Germany by Ullerich (1966) confirmed the karyotype of 2n = 22 chromosomes, all metacentric except for the submetacentric 4th and 8th pairs. These investigations described a secondary constriction terminal to the long arms of chromosomes of the 6th pair and denied the presence of heteromorphic sex chromosomes. This chromosome formula was confirmed in European pet trade toads of unknown origin by Schmid (1978), in the first of his long, very interesting, series of papers on chromosome banding in Amphibia. This author confirmed the presence of NORs peritelomeric on the long arms of the 6th pair and provided the pattern of C-banding positive heterochromatin (see Fig. 2). Similar C-banded karyotypes were displayed by Serbian (Spasić-Bosković et al., 2000) and Russian (Matsui et al. 2013) specimens (see Fig. 2 for the corresponding ideograms). Furthermore, chromosome analysis conducted on taxa formerly considered as subspecies of *B. bufo*, namely *B. spinosus*, *B. verrucosissinus*, *B. japonicus* and *B. eichwaldi*, showed that they conserved the localization of NORs peritelomeric on the long arms of the chromosomes of the 6th pair and each exhibited a distinctive pattern of C-banding positive heterochromatin (Birstein and Mazin 1982; Matsui et al. 1985; Skorinov et al. 2014; Guzmán-Markevich et al. 2022). All exhibited centromeric C-bands on all chromosomes but differed on the pattern of paracentromeric, interstitial and/or telomeric heterochromatin. In Urodeles evidence from studies on satellite DNAs (Hutchinson and Pardue 1975; Macgregor and Sessions 1986; Macgregor 1991), which are a major component of heterochromatin (John 1988), proposed the following evolutionary steps on the chromosome

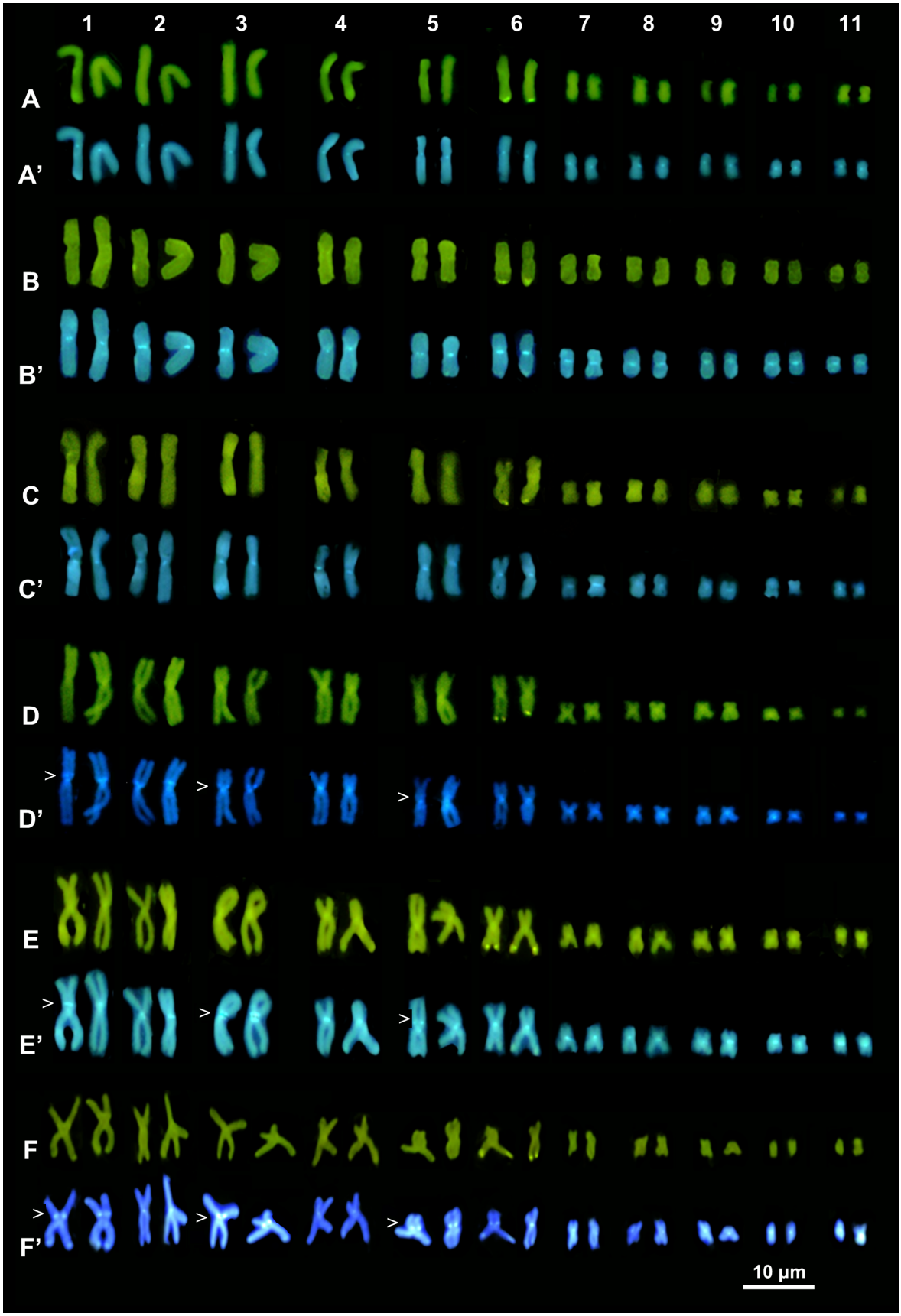

Figure 1: Karyotypes stained with sequential C-banding + CMA (**A**-**F**) + DAPI (**A'-F'**) of specimens of *B. bufo* from: Conero (Marche) (**A**, **A'**); Minturno, Rio Santa Croce, Lago Penitro (Lazio) (**B**, **B'**); Lago Letino, Gallo (Campania) (**C**, **C'**); Altamura (Puglia) (**D**, **D'**); Agnone Cilento, Montecorice, Corleto (Campania) (**E**, **E'**); San Nicola Arcella (Calabria) (**F**, **F'**). Bar refers to all karyotypes. > point to paracentromeric, DAPI positive C-bands.

heterochromatin distribution: i) an initial amplification of satellite DNA arrays occurring at centromeric/ pericentromeric regions; ii) intrachromosomal rearrangements satellite sequences on interstitial and/or telomeric regions, with some arrays as remnants of the original amplified satellite sequence still evident on centromeric C-bands. The results of C-banding staining here obtained show a different pattern of heterochromatin between population of common toad from central (Ancona, Latina + Caserta) and southern (Salerno and Cosenza) populations. In turn, the C-banding patterns of above population also differ from those available in literature, which concerned populations from Serbia, near Moscow and an unknown European population (Schmid 1978; Birstein 1982; Spasić-Bošković et al. 2000), that we have superimposed on the image (opportunely modified) of the distribution areas of clades of *B. bufo* by Garcia-Porta et al. 2012 (Fig. 2).

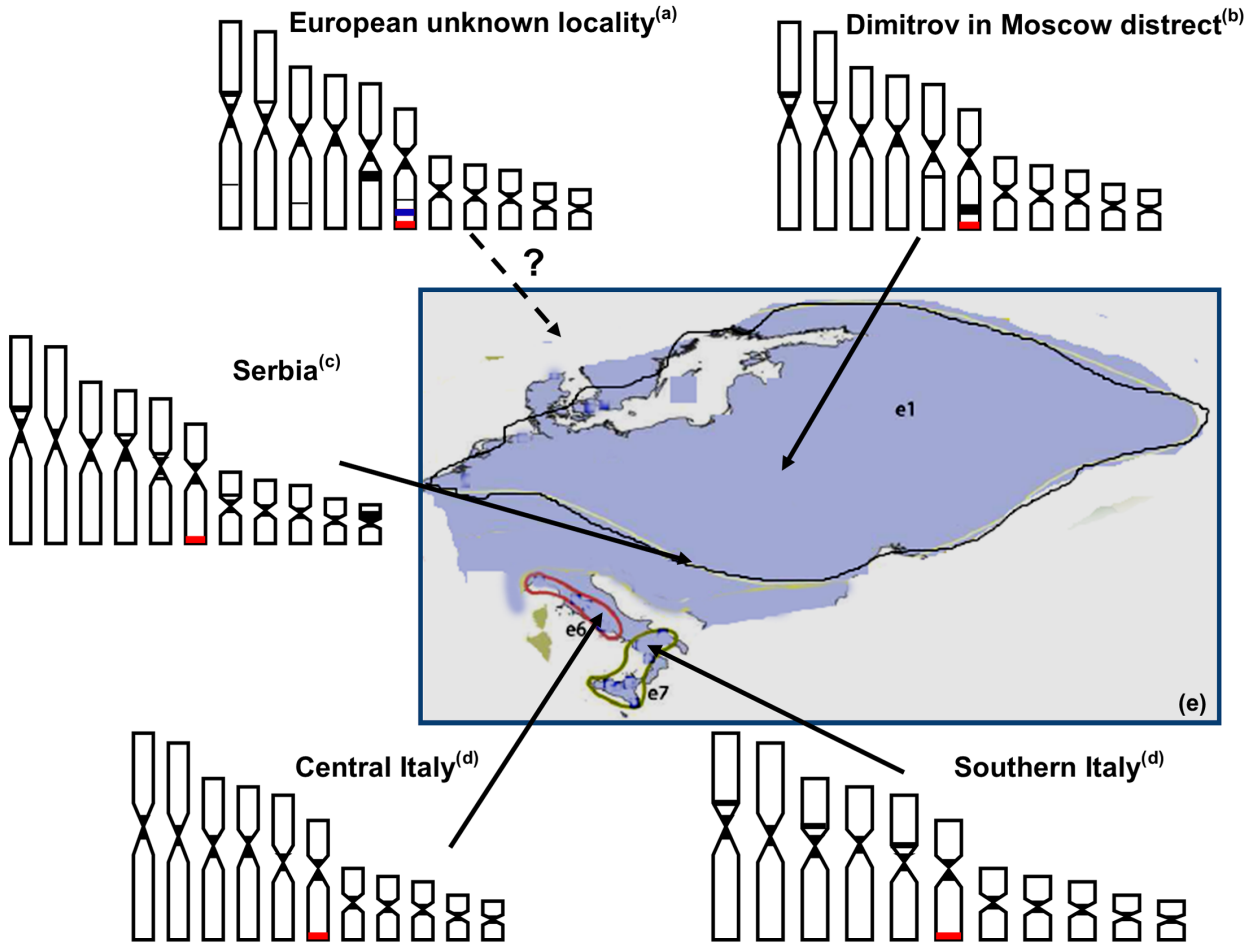

Figure 2: Schematic haploid karyotype ideograms of populations of *B. b. bufo* superimposed on the geographic distribution of its clades e1, e6 and e7 according to: Garcia-Porta et al. (2012); Schmid 1978 (a); Birstein 1982 (b); Spasić-Bošković et al. 2000 (c); present paper (d). The Figure was modified from Garcia-Porta et al. (2012). Dark, blue and red bands refer, respectively, to C-banding positive heterochromatin, NOR-associated heterochromatin and Loci of NORs.

Interestingly, the central and southern Italian populations are in the clades e6 and e7, respectively, while the Russian and Serbian population are in the clade e1. Note, that the Serbian population are in southern margin of distribution of the e1 clade, leaving uncertain its attribution at this clade. So, C-band patterns reveal a population variability regarding heterochromatin distribution, which in turn appear to support/discriminate the distribution areas of clades e6 and e7 of *B. bufo* (Garcia-Porta et al. 2012). However, some cautions should have to consider C-bands, specifically the subtle interstitial ones. These bands may be not reproducible or not visible, due to methodological differences among laboratories to prepare, which are in more condensed chromosomes, as by preparation from cell cultures or from testis. In any case solid paracentromeric and or interstitial C-bands are independent from the employed methodology and tissues to obtain chromosomes. So, they can be used as landmarks to discriminate the different populations of *B. bufo*. Furthermore, the chromosome evidence here obtained supports the hypothesis advanced by Chiocchio et al. (2021) that the Central and Southern Italy lineages of *B. bufo* expanded form their ancestral glacial refugia along Tyrrhenian coastal refugia, with the Volturno-Calore rivers lower basin as suture zone, establishing a secondary contact zone before the last interglacial.

In conclusion, the chromosome C-banding patterns seem useful in distinguishing taxonomically different forms of *B. bufo*, but their use in relation to speciation events or systematic relationships need further analysis.

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