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Short Communication

How complex is the *Bufo bufo* species group? ☆

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ABSTRACT

Species delineation remains one of the most challenging tasks in the study of biodiversity, mostly owing to the application of different species concepts, which results in contrasting taxonomic arrangements. This has important practical consequences, since species are basic units in fields like ecology and conservation biology. We here review molecular genetic evidence relevant to the systematics of toads in the *Bufo bufo* species group (Anura, Bufonidae). Two studies recently published in this journal (Recuero et al., MPE 62: 71–86 and García-Porta et al., MPE 63: 113–130) addressed this issue but reached opposing conclusions on the taxonomy of the group (four versus two species). In particular, allozyme data in the latter paper were interpreted as evidence for hybridization across species (between *B. bufo–B. spinosus and B. bufo–B. verrucosissimus*). We tested claims for hybridization through re-analysis of allozyme data for individuals instead of populations, to be able to distinguish between sympatry with and without admixture, and found no evidence of hybridization across taxa. We propose alternative explanations for the observed patterns that García-Porta et al. (2012) failed to consider. In the absence of unequivocal evidence for hybridization and introgression, we reject the proposal to downgrade *Bufo spinosus* and *Bufo verrucosissimus* to the subspecies level.

1. Introduction

Species diagnosis and delineation remain among the most challenging tasks in the study of biodiversity. This is an old problem that has not been solved by our recent ability to integrate massive amounts of – especially molecular – data; the challenge seems only to get bigger. At the core of the problem is the coexistence of different species concepts, whose alternative application results in contrasting taxonomic arrangements. This has important consequences since species are the basic units in diverse fields like ecology, evolutionary biology, and conservation biology. For instance, species are the basic categories in global management policies like IUCN's Red List, and both over- and underestimation of species numbers can be detrimental to conservation efforts. In this paper, we review available molecular evidence relevant to the systematics of toads in the *Bufo bufo* (*sensu lato*) species group (Anura, Bufonidae). Two studies published in this journal last year (Recuero et al., MPE 62: 71–86 and García-Porta et al., MPE 63: 113–130) addressed this issue but reached opposing conclusions on the taxonomy of the group (four versus two species), despite overall congruence across datasets. We address these discrepancies explicitly advocating a species concept in which species are lineages of ancestral-descendant populations that have evolved separately from other lineages long enough to acquire diagnostic differences and an inability to merge upon secondary contact, consistent with the phylogenetic species concept (*sensu* Cracraft, 1983). That is, if two different species lineages have diagnosably distinct evolutionary histories and hybridize only to a limited amount at their borders, they would constitute separate species. On the other hand, populations that never experienced a long history of evolution in isolation from each other, and whose geographic populations have been separated only by "isolation by distance" through their evolutionary histories would be considered a single species.

The Common toad species group of the western Palearctic consists of four species, namely *Bufo bufo* (Linnaeus, 1758), *Bufo eichwaldi* Litvinchuk, Borkin, Skorinov and Rosanov, 2008, *Bufo spinosus* Daudin, 1803 and *Bufo verrucosissimus* (Pallas, 1814). The species' approximate ranges are described in the legend of Fig. 1. Molecular genetic data on two mitochondrial genes (1239 bp) and four nuclear genes (2251 bp) yielded a robust phylogeny in which the basal split separates *Bufo eichwaldi* from the other species, followed by *Bufo spinosus* splitting from a common

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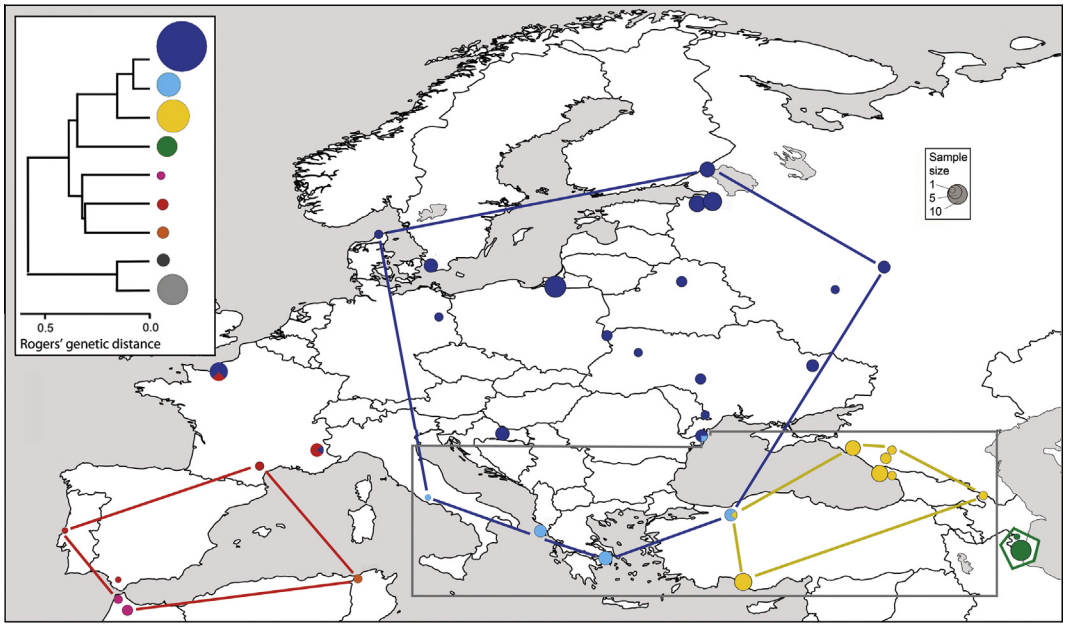

Fig. 1. Summary of allozyme results in the *Bufo bufo* species group. The *Bufo bufo* species group is composed of *Bufo spinosus* from northern Africa, the Iberian peninsula and southern France, *Bufo bufo* with a wide range from northern France to deep in Russia, in southern Europe extending over the Apennine and Balkan peninsulae, *Bufo verrucosissimus* from the western Caucasus and *Bufo eichwaldi* from the Talysh Mountains in south Azerbaijan and Iran. The convex polygons encompass genotype clusters that are derived from 16 polymorphic enzyme loci with Bayesian clustering. Thirty-six investigated populations are shown by circles in which the diameter is representative of sample size (see box with "sample size" for scale). Colours indicate species allocation as follows: three shades of red – *Bufo spinosus*, two shades of blue – *B. bufo*, yellow – *B. verrucosissimus* and green – *B. eichwaldi*. The outgroup *B. gargarizans* ($n = 31$ in four populations) falls outside the map. To be noted are the mixed "blue–light blue" *B. bufo* locality near Odessa, Ukraine and the sympatric occurrence of *B. bufo* and *B. verrucosissimus* at Lake Abant in northwestern Turkey. For details on the sympatric occurrence of *B. bufo* and *B. spinosus* mtDNA haplotypes in northwestern and southeastern France see Fig. 3. Twelve populations from the boxed area are examined for *B. bufo* – *B. verrucosissimus* inter-specific hybridization (as in Fig. 2). Insert – UPGMA dendrogram of nine BAPS genotype groups on the basis of Rogers' genetic distance (Swofford and Selander, 1981). The outgroup *B. gargarizans* is shown by two shades of gray and circles are proportional to sample size. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ancestor of the sister-species *Bufo bufo* and *Bufo verrucosissimus* (Recuero et al., 2012). A coalescent-based estimation of the time to most recent common ancestry allowed a historical reconstruction of the diversification of the group in the context of Mediterranean paleogeography. A congruent topology derived from three mitochondrial genes (1988 bp) was subsequently reported by García-Porta et al. (2012), although clades were allocated to regions rather than to species, with the following synonyms: European = *Bufo bufo*, Caspian = *Bufo eichwaldi*, Iberian and African = *Bufo spinosus* and Caucasian = *Bufo verrucosissimus*. The latter two clades these authors consider to be subspecies of *Bufo bufo*. The most recent common ancestor of these four species is estimated at ca. 13.1 Ma (millions of years) by Recuero et al. (2012) and at ca. 7.4 Ma by García-Porta et al. (2012). The difference is due to the calibration points sought to fix the tree in time, but both estimates indicate a long independent evolutionary history of the lineages in this group.

In spite of the deep genetic distinction based on mtDNA and nDNA sequence data, García-Porta et al. (2012) reported evidence for hybridization of *B. bufo* and *B. spinosus* in the southwest of Europe and for *B. bufo* and *B. verrucosissimus* in southeastern Europe. With no sympatry of mtDNA or nuclear haplotypes and with no data on morphological intergradation, the case rests solely with their allozyme data. We here scrutinize claims for hybridization through a data re-analysis for individuals instead of populations, e.g. to be able to distinguish between sympatry with and without admixture. We propose explanations other than hybridization that García-Porta et al. (2012) failed to consider. In the absence of unequivocal evidence for hybridization and introgression, we reject the proposal to downgrade *Bufo spinosus* or *Bufo verrucosissimus* to the subspecies level.

2. Materials and methods

Individual genotype data are available for 21 enzyme loci scored for 173 toads in 40 populations and five species (S. Litvinchuk, pers. comm.). Average sample size per population was 3.9 for the ingroup (36 populations, range 1–12) and 7.8 for the outgroup (*Bufo gargarizans*, four populations, range 4–18). 1.7% of data were missing. We used a Bayesian analysis of population structure with the program BAPS 6 (Corander et al., 2004). BAPS assigns individuals to distinct gene pools probabilistically, based upon multilocus genetic data, where each individual allele is coded at two alleles per locus. BAPS does not make a priori assumptions about the number of gene pools (k), and we let BAPS determine the most probable value of k over the 1–20 range, under default parameter settings. Populations were assessed for interspecific hybridization with Flock 3.0 (Duchesne and Turgeon, 2009) and NewHybrids software (Anderson and Thompson, 2002), also under default settings. F_{ST} calculations were done with FSTAT 2.93 (Goudet, 1995).

We also re-analyzed the nuclear sequence data of Recuero et al. (2012). Two haplotypes per individual were phased for each of the genes BDNF, CXCR4, POMC and RPL3 using SeqPHASE (Flot, 2010) and PHASE2.1.1 (Stephens et al., 2001). Haplotype networks were constructed with HaploViewer (available at http://www.cibiv.at/~greg/haploviewer) using a neighbor-joining tree reconstructed with PAUP (Swofford, 2001).

3. Results and discussion

3.1. Current taxonomic status

The species status of *B. bufo* and *B. eichwaldi* is unchallenged, but what is the situation for *B. spinosus* and *B. verrucosissimus*? *Bufo*

spinosus was elevated to the species level by Recuero et al. (2012) and kept as a *B. bufo* subspecies by García-Porta et al. (2012), whereas *B. verrucosissimus* was kept as a species by Recuero et al. (2012) and coined a subspecies by García-Porta et al. (2012). The search term "verrucosissimus" returned ca. 15 000 hits in Google (accession date January 5, 2012), for which, in the top one-hundred, 99 hits referred to ' *Bufo verrucosissimus*' and one to "Bufo bufo verrucosissimus" (this was Tosunoğlu and Taskavak, 2001). Google Scholar and the Ovis digital version of the Zoological Record look deeper in time. The same search term yielded 58 literature references of which 41 (71%) concerned "Bufo verrucosissimus" and 17 (29%) were on "Bufo bufo verrucosissimus" (a recent citation is e.g., Sinsch et al., 2009). We observed a change from subspecies to species status over two decades ago and allocate this event to the paper by Orlova and Tuniyev (1989). We point out that "verrucosissimus" as a species is the *status quo*, and whether correct or not, the burden of proof is with those suggesting change and we here argue that convincing data that warrant a change in taxonomy have not yet been put forward.

3.2. Population genetic structure

The Bayesian analysis of enzyme profiles places the 173 individuals into nine genotype groups. These groups are organized in five geographically and hierarchically coherent units conforming to the five species as follows: *B. spinosus* with three groups (Iberia and France, Morocco, Tunisia), *B. bufo* with two groups (northern and southern), *B. verrucosissimus* and *B. eichwaldi* with one group each, and two for the outgroup species *B. gargarizans* (Fig. 1). The enzyme genetic distances between the species are substantial (Fig. 1, insert), in line with the high level of divergence at mtDNA and nDNA loci (García-Porta et al., 2012; Recuero et al., 2012). Observations on species (or their genetic markers as the case may be) in sympatry are as yet limited to Lake Abant for *B. bufo* and *B. verrucosissimus* and to *B. bufo* and *B. spinosus* in northwestern and southeastern France (Fig. 1).

Prior to evaluating claims for hybridization, we point to alternative interpretations for "population intermediacy" that were not yet considered. Firstly, ancestral polymorphisms with incomplete sorting of alleles through the divergence of populations can produce the same pattern. Whereas *B. bufo* and *B. verrucosissimus* share nuclear DNA haplotypes at some loci, there is no geographic pattern in allele sharing, suggesting that incomplete lineage sorting is a more plausible explanation than hybridization. Secondly, the "intermediate" populations (45, 69, 108 and 117 in García-Porta et al., 2012) are all from southern Europe and positioned within the area of glacial refugia proposed by these and other authors. A more or less intermediate position of southern populations in either a multivariate or a tree-based analysis (García-Porta et al., 2012: Fig. 4) is, in our opinion, just what is expected under Hewitt's (2000) "southern richness – northern purity" paradigm. Accordingly we argue that it is not the southern populations that show surprisingly high genetic variation (e.g., from hybridization and introgression), but that the northern populations show uniform, low genetic variation, due to a loss of alleles during postglacial expanse. The southern genetic richness is therewith not due to secondary contact but to long term *in situ* evolution. The southern populations retained the ancestral geographic location and a larger portion of the ancestral variation than do other populations. We suggest that the highlighted populations are not "intermediate" and it is then peculiar that the one genuinely mixed Lake Abant population was not identified as such.

3.3. Diagnostic value of enzyme loci and evidence for hybridization

Among 21 enzyme loci studied 16 were polymorphic. For *B. bufo* and *B. spinosus* the global F_{ST} over 13 polymorphic loci was 0.79 (95% confidence interval 0.63–0.89). Six loci with an F_{ST} in excess of 0.8 were *G6dp-1*, *Gtdp-2*, *Ldh-1*, *Ldh-2*, *Sod-1* and *Sod-2*. However, we refrained from re-analyzing *B. bufo* and *B. spinosus* hybridization and introgresion from allozyme data because the study populations are far apart (ca. 900 km over land) and sample sizes are small. For the combination of *B. verrucosissimus* and southern *B. bufo* (light blue in Fig. 1, the Lake Abant population excluded) the global F_{ST} over ten polymorphic loci was 0.54 (95% confidence interval 0.09–0.84). Two loci with an F_{ST} in excess of 0.8 were *Xdh-1* (F_{ST} = 1.0, i.e., fully diagnostic) and *G6pd-1* (F_{ST} = 0.86). Allele frequencies at the latter locus were 0.75 at *G6pd-1*^{a}, 0.10 at *G6pd-1*^{b} and 0.15 at *G6pd-1*^{c} in *B. bufo* and 1.0 at *G6pd-1*^{c} in *B. verrucosissimus*.

The enzyme data for *B. bufo* and *B. verrucosissimus* concern 45 individuals in 12 populations in a transect from Italy to the Caspian Sea, as shown in the boxed area of Fig. 1. Analyzed with a Bayesian approach (NewHybrids, P_{NH}), all individuals fall outside the 0.1–0.9

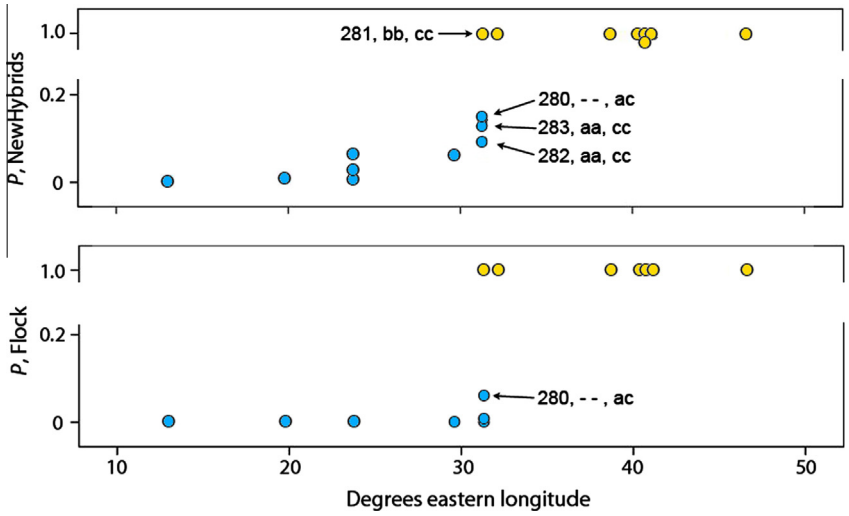

Fig. 2. Enzyme identification of potential hybrids between *Bufo bufo* and *B. verrucosissimus*. Plotted is the probability (*P*) of individual toads to be identified as *B. verrucosissimus* versus *B. bufo* with NewHybrids (top panel, P_{NH}) and Flock 3 software (lower panel, P_F represents normalized likelihood score) in a transect from southern Europe (*Bufo bufo* – light blue) to the Caucasus (*B. verrucosissimus* – yellow). Individuals from Lake Abant are positioned at eastern longitude 31° 17', with their number in the original data base and the alleles observed at the loci *Xdh* and *G6pd-1*. Note that individual 280 has not been scored for the one fully diagnostic locus *Xdh-1*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

range, with the exception of the toads 280 and 283 from Lake Abant (Fig. 2). Similarly, with a re-allocation method all individuals had normalized likelihood scores outside the 0.01–0.99 range (Flock, P_F), with the exception of individual 280 from Lake Abant. The signal for genetic admixture is weak and, in the Flock results, only apparent when data for the single fully diagnostic locus *Xdh-1* are missing. So, as before, the sample of Lake Abant in northwestern Turkey is composed of one *B. verrucosissimus* and three *B. bufo*. Natural hybrids between these species have yet to be documented. The experimental crossing of *B. bufo* and *B. verrucosissimus* yielded fertile F_1 hybrids and their back-crossing with *B. bufo* yielded unfertilized eggs (one time) and developing embryos (two times; Pisanets, 2002; Kuzmin, 2012).

The combined data (allozymes, mtDNA, nDNA) point to a complicated, perhaps mosaic distribution in Turkey with, from west to east, *B. bufo* in European Turkey and south of the Sea of Marmara, *B. bufo* and *B. verrucosissimus* in sympatry at Lake Abant, *B. bufo* in central Turkey and *B. verrucosisimus* in the east (Fig. 3). A mosaic distribution of *B. bufo* and *B. verrucosissimus* would be compatible with parapatric range borders, in which the taxa are distributed according to characteristics of the environment (e.g., soil type), with limited hybridization at sharp contact zones. Interestingly, northwestern Turkey was also identified as a zone of complexity in several species of newts (Nadachowska and Babik, 2009; Arntzen and Wielstra, 2010; Wielstra et al., 2010, 2013). Another possibility is the presence of *B. bufo* mtDNA at the nuclear background of *B. verrucosissumus*. This phenomenon is not rare and may even make an entire species invisible from the mtDNA perspective (Zieliński et al., 2013).

3.4. Diagnostic value of nuclear sequences and evidence for hybridization

The networks for nuclear genes confirm a close association of *B. bufo* and *B. verrucosissimus* haplotypes (Fig. 4). Under the poor sampling of the latter species we can, however, not distinguish between incomplete lineage sorting and recent hybridization as the process underlying this result. *Bufo bufo* and *B. spinosus* are fully separated at two loci (BDNF and RPL3), indicating the absence of evidence for hybridization under their sampling regime. In a third marker, POMC, a single allele was shared between the species in one toad in northern France (locality Erloy, BB141 in Recuero et al., 2012) and the possibility of introgression cannot be excluded (a more detailed study is in preparation). Finally, to explain the observed allele sharing at CXCR4 we consider incomplete lineage sorting a better explanation than recent hybridization because even outgroup alleles are found associated with the ingroup and because of the absence of a spatial signal in allele sharing (results not shown).

3.5. Contrasting interpretations

The individual-based analysis of enzyme data identified the Lake Abant population in northwestern Turkey as *B. bufo* and *B. verrucosissimus* in sympatry with no unequivocal evidence for hybridization or introgression. Conversely, in a population-based analysis, García-Porta et al. (2012: page 127 and Fig. 4) report on introgressive hybridization, but they position the area of inter-specific gene flow across Greece: "The results of the MCA analyses [Multiple Correspondence Analysis] ... and a close inspection of the allozyme frequency table ... clearly show that some of the populations present a mixed ancestry indicating extensive past or ongoing introgression. For instance, Greek populations 69 and 117 ... have an intermediate position between the Caucasus populations and the European populations in the MCA allozyme analysis". On *B. bufo – B. spinosus* they continue: "The same occurs with specimens [sic, $n = 1$] from locality 108 ... with some Iberian alleles at some loci ... or with [$n = 2$] specimens from locality 45, classified as belonging to the Iberian population ... but with

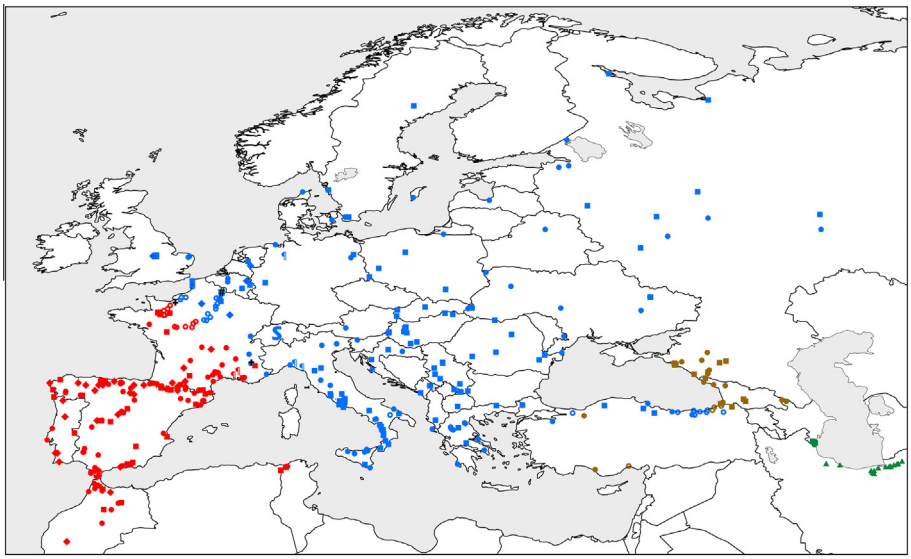

Fig. 3. Distribution data of species in the *Bufo bufo* species group. Documented localities of *Bufo* species in Europe and north Africa, with *Bufo spinosus* in red, *B. bufo* in blue, *B. verrucosissimus* in brown and *B. eichwaldi* in green. Data sources are: Kutrup et al. (2006) open round symbols in Italy and Turkey, Recuero et al. (2012) square symbols, García-Porta et al. (2012) solid round symbols, Litvinchuk et al. (2012) triangle symbols. The "S" stands for 28 *B. bufo* populations from in and around Switzerland that were studied by Lüscher et al. (2001) while five other of their populations are shown by the '¶' symbol. For Far-eastern *B. bufo* localities see Recuero et al. (2012). Additionally shown are data from Arntzen et al. (submitted for publication) with diamond symbols for museum material and open round symbols in France for genetic data; two French localities with *B. bufo* and *B. spinosus* genetic markers in sympatry are indicated by a black cross. The locality Erloy in Northern France where *B. bufo* and *B. spinosus* specific alleles of the gene POMC are found in sympatry is shown by the '#'symbol. Partial 16S ribosomal RNA sequences by Kutrup et al. (2006) are allocated to *B. bufo* and *B. verrucosissimus* on the basis of a single, locally diagnostic nucleotide ('T' in *B. bufo* and 'C' in *B. verrucosissimus*), homologous to position 4597 of the *Bufo japonicus* mitochondrial genome (Igawa et al., 2008). Note the discrepancy with the nuclear genetic species identifications in Fig. 1 in which the convex polygon of *B. verrucosissimus* encompasses populations in northern Turkey that are here, from various mitochondrial genes, identified as *B. bufo*. For the range border of the *B. bufo – B. spinosus – B. verrucosissimus* group across Eurasia see Sinsch et al. (2009); for the distribution of *B. spinosus* in Northern Africa see Beukema et al. (2013) and Bogaerts et al. (2013). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

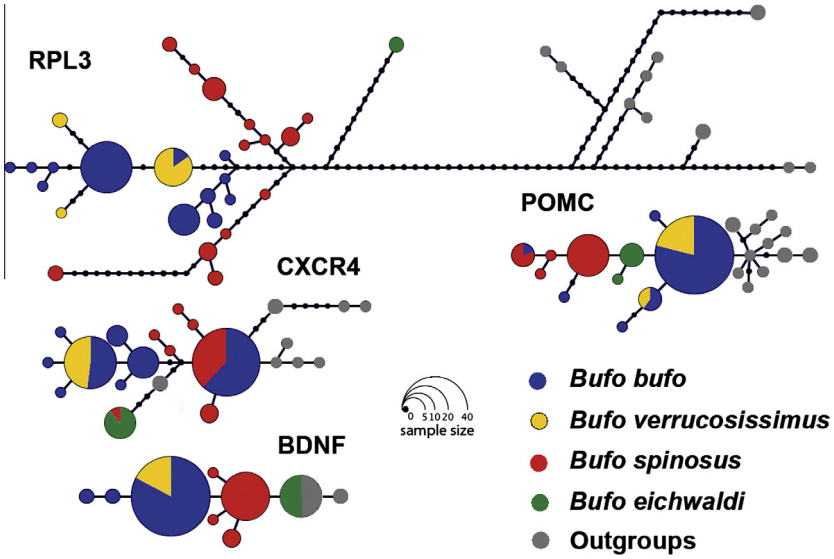

Fig. 4. Haplotype networks for four sequenced nuclear genes in the *Bufo bufo* species group (data Recuero et al., 2012). Note the shared alleles between *B. bufo* and *B. verrucosissimus* in all markers and in POMC and CXCR4 for *B. bufo* and *B. spinosus*.

European alleles in some loci . . .". Finally, on the basis of "observed introgression", García-Porta et al. (2012) prefer to regard the Caucasian [*B. verrucosissimus*], European [*B. bufo*], Iberian and African [*B. spinosus*] clades as subspecies of *Bufo bufo*. We do not share these preferences because the case for introgressive hybridization was nowhere justified and alternative explanations such as incomplete lineage sorting and "southernrichness" were not considered.

3.6. Taxonomic consequences

We elevated *B. spinosus* to the species level (Recuero et al., 2012). This proposal is based upon, first, a deep genetic differentiation with no evidence for hybridization. In particular, the enzyme data of García-Porta et al. (2012) yield no information that would challenge this assessment. Second, in the molecular phylogenies (3490 bp of concatenated mt and nDNA data) and in the analyses of allozyme data (Fig. 1), *B. bufo* and *B. spinosus* are not sister-taxa. Third, once looked for at the right place, morphological differences between *B. bufo* and *B. spinosus* are small but consistent with no wide zone of intergradation, at least not at the center of one broad latitudinal transect from the UK to Morocco (Arntzen et al., submitted for publication). While *Bufo bufo* and *Bufo spinosus* are morphologically distinguishable, the differences in phenotype are small compared to the level of genetic differentiation. *Bufo bufo* and *Bufo spinosus* therewith classify as "cryptic species" (Arntzen et al., submitted for publication).

In the case of *B. verrucosissimus*, individual-based analyses reject hybridization as the cause of allele-sharing across taxa. As yet the nature of the mutual species-range border is unclear. Along with Kuzmin (2012), we acknowledge that further work is required to elucidate the taxonomic status of *B. verrucosissimus* and that, until proposals for change are well supported, *B. verrucosissimus* is maintained as a species to preserve stability of nomenclature.

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