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A common toad hybrid zone that runs from the Atlantic to the Mediterranean

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Abstract. We document the distribution of the common toad *Bufo bufo* and the spined toad *B. spinosus* at their contact zone across France with data from a mitochondrial DNA RFLP assay, complementing similar work including nuclear markers in the northwest and southeast of France and in Italy. We also reconstruct geographical clines across the species' contact zone in central France. *Bufo bufo* is found in the north-eastern half of France. *Bufo spinosus* is found in the south-western complement. The contact zone they form runs from the Atlantic coast near Caen, France, to the Mediterranean coast near Savona, Italy, and has a length of over 900 km. In central France *B. bufo* and *B. spinosus* engage in a hybrid zone with a unimodal genetic signature. Hybrid zone width is ca. 10 km at mitochondrial DNA and averages at 61 km for four nuclear loci. The hybrid zone is distinctly asymmetric with a signature of *B. spinosus* in *B. bufo* and not the other way round. We attribute this observation to *B. bufo* moving southwards at the expense of *B. spinosus*, with introgression in the direction of the advancing species. We noted substantial geographic variation in characters for species identification. Morphological species identification performs well in France, but breaks down in Italy. Mitochondrial DNA is inconclusive in south-eastern France and Italy. The nuclear genetic markers perform consistently well but have not yet been applied to the zone in full. Possible, but surely heterogeneous ecological correlates for the position of the hybrid zone are mountains and rivers.

Keywords: *Bufo bufo*, *Bufo spinosus*, France, geographical cline, geographical variation, Italy, mitochondrial DNA, RFLP.

Introduction

Hybrid zones have long been regarded as natural laboratories for the study of speciation (Hewitt, 1988). Inspired by the review of Hewitt (2000), many studies have further documented the location of secondary contact zones in a wide range of hybridizing taxa across Europe (for reviews see Taberlet et al., 1998; Habel et al., 2010; Schmitt and Varga, 2012). This has revealed general patterns of concordance in the location of so-called suture zones, especially among taxa surviving the Pleistocene Ice Ages in different southern European peninsulas, that later expanded out of their refugia to colonize previously unsuitable areas in central and northern Europe. The addition of data from new model systems builds on previously compiled comparative data and helps to address questions related to the timing and the spatial and ecological context of hybrid zone formation.

One emerging model system is formed by the common toad (*Bufo bufo*) and the spined toad (*B. spinosus*), which are genetically deeply differentiated yet morphologically similar species that meet up in France (Recuero et al., 2012; Arntzen et al., 2013a). They are not sister species. Once their evolutionary independence had become apparent, a search for morphological features that would allow species identification in the field pointed to adult body size, the positioning of the parotoids and the size and shape of the metatarsus tubercle as species diagnostic characters (Arntzen et al., 2013b). Based on these morphological features, which were consistent with species assignment based on molecular data, we broadly delineated their contact zone in France, from near Caen in the northwest to Lyon in the southeast. The contact zone encompasses great topographic (and thus ecological) heterogeneity, potentially condition-

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ing the initial location, dynamics and outcome of the species contact. Therefore, a finer-scale delineation of the zone can reveal the role of topographic and ecological factors in shaping the contact between the two species.

We have previously shown that *B. bufo* and *B. spinosus* do hybridize (Trujillo et al., 2017) and that, in the northwest of France, they do so in a narrow zone (Arntzen et al., 2016). Cline analyses located the centre of the contact zone at the northern slope of the 'Collines de Normandie' (Arntzen et al., 2016). In this region, mtDNA is largely co-distributed with nuclear markers and with morphology in defining species borders, and thus mtDNA genotyping can possibly be used as a fast, cost-effective approach to accurately delineate the contact zone in the remainder of the area where the two species meet. Here we use new and published molecular data to document the precise position of the toad hybrid zone from the Atlantic to the Mediterranean coast. We discuss the role of major rivers and topographic features in shaping species boundaries and their interactions. This information can in turn be used to subsequently select and define replicated transects on which to perform more detailed analyses of the hybrid zone, using genomic data in a cline analysis framework. Additionally, the mapping of the contact zone allows fine-scale delineation of the ranges of the two species, which is a basic step required to complete global or regional assessments on their conservation status.

Materials and methods

DNA was extracted from 1428 tissue samples in 140 populations ($\tilde{N} = 10.2$, range 1-40). For a visualisation of the sampling effort see online supplementary fig. S1 and supplementary .kml file and for locality information see table 1. Species diagnostic mtDNA haplotypes were determined with an RFLP-assay on the cytochrome-b gene following Arntzen et al. (2013b). In six cases (0.4%) the RFLP banding pattern was vague and no call was made. For four individuals in two populations (B011 and B065) where species assignment based on RFLP profiling resulted in 'geographic outliers', outside the inferred species' range, the DNA sequence of a 722 bp sized fragment of cytochrome-b was determined as in Recuero et al. (2012), for cross-checking. To find identical or similar sequences they were 'blasted' at Genbank (https://blast.ncbi.nlm.nih.gov/).

We studied the *B. bufo*-*B. spinosus* species transition in central France in a transect from the southwest to the northeast under a 45-degree angle. The transect was directed through populations B137 and B102 and positioned roughly perpendicular to the mitochondrial DNA contact zone (see fig. 1). Initially 15 populations were analysed for two mtDNA markers (cytochrome-b and 16S) and four nuclear markers (BDNF, POMC, RAG1 and RPL3). Results indicated that there is no significant tail at the *B. spinosus* side of the transect for either of the genetic markers. Conversely, substantial tails were observed at the *B. bufo* side for all nuclear markers. We therefore extended the transect northwards with populations from Belgium (B472-476) and the Netherlands (B171, B172-173 and B359). Altogether 19 populations were studied for mtDNA SNPs with an average sample size of $N = 10.7$ (range 6-28) and with 0.5% of missing data. The same populations were analysed for the four nuclear SNPs with an average sample size of $N = 10.0$ (range 2-28), with 2.1% missing data. Analysis followed the procedures described in Arntzen et al. (2016). The molecular data that describe the species transition were analysed with the geographical cline fitting software HZAR (Derryberry et al., 2014) as described in Arntzen et al. (2017a).

Results

The inferred mtDNA haplotype identities (table 1) are plotted per population as *B. bufo*, *B. spinosus* or both species in fig. 1. For ease of interpretation the results are presented in Dirichlet cells for which the spatial extrapolation does not exceed ca. 40 km. The *B. bufo*-*B. spinosus* contact zone runs across France from near the mouth of the Seine river at the Atlantic coast till near Grenoble at the French Alps. Over those parts of France where the sampling is dense, the width of the area with both species rarely exceeds 50 km. The cytochrome-b sequence for one RFLP outlier data point indicating *B. spinosus* in the north was actually equivalent to the *B. bufo* sequence JN647248, whereas three outlier data points indicating *B. bufo* in the south were equivalent to the *B. spinosus* sequence JN647327. It thus appears that these outliers correspond to identification errors caused by homoplasious nucleotide substitutions.

In the southeast of France our sampling is thin, the mutual species distribution appears a mosaic and contact zone position and width are

Table 1. Common toad populations studied for mitochondrial DNA species affiliation, with coordinates and sample sizes. Populations that make up the transect in central France are marked with a #. Populations additionally studied are listed at the bottom. For a map with localities plotted see supplementary fig. S1 and supplementary .kml file.

Population number	Locality – note	Northern latitude	Eastern longitude	mtDNA haplotype typical for	
				Bufo bufo	*Bufo spinosus*
B000	Autreppes – data from Arntzen et al. (2016)	49.915	3.847	28	0
B001	Sorques	48.345	2.778	11	0
B002	Audresselles	50.821	1.602	22	0
B003	Camiers	50.574	1.608	8	0
B005	Southeast of Berck	50.399	1.606	7	0
B009	Between Gamaches and Monthieres	49.969	1.584	9	0
B011	Bord des Bois – one individual sequenced	49.782	1.606	7	1
B012	Cleres	49.597	1.111	6	0
B013	Jublains, carrefour Poteau	48.240	−0.552	0	26
B018	Foret Domaniale des Andaines	48.595	−0.553	0	8
B019	Fromentel	48.726	−0.273	0	8
B020	Vendeuvre	48.990	−0.072	0	8
B021	Moyaux	49.199	0.327	25	9
B022	Mare Bouttieux	49.435	0.704	8	0
B025	Bois de la Grange	48.730	3.378	5	0
B026	La Blandinerie, NE of Bois de la Main Ferme – one individual sequenced	48.924	3.378	5	0
B027	l'Etang de la Logette	49.119	3.499	5	0
B028	Fontaine du Prince, Dominale de Retz	49.254	3.179	5	0
B030	Etangs de Comelle	49.158	2.500	15	0
B033	Etang de Changy	47.854	2.678	9	0
B035	Etang de la Tour	48.661	1.880	23	0
B036	Etang Neuf, Villebon	48.389	1.200	6	4
B037	Etang du Gré, Foret Dominiale du Perche	48.623	0.625	2	9
B038	Etang de la Herse	48.403	0.549	0	8
B039	SW of Ailliéres-Beauvoir	48.395	0.313	0	22
B040	l'Etang Neuf, next to D21	48.334	0.005	0	8
B041	South of Pré-en-Pail	48.447	−0.201	0	8
B042	Étang de Vrigny	48.654	−0.021	0	8
B043	Foret Communale du Bourg-st-Léonard	48.769	0.098	0	8
B044	Farm NE of Roiville	48.884	0.251	0	12
B045	La Grosse Forge, NE of Ferriéres-st-Hilaire	49.040	0.576	8	4
B047	Dominiale de Roumare	49.442	0.995	8	0
B048	Abbaye de Mortemer	49.369	1.481	5	0
B050	Étang des Moines, Genainville	49.121	1.760	7	0
B051	Mare de Prince, Foret de Bizy	49.070	1.452	8	0
B052	Chenappeville	49.001	1.099	18	0
B053	Between St-Germain-sur-Avre and Vert-en-Drouais	48.759	1.270	11	0
B055	Bassins Mousseuse	48.612	0.892	1	8
B056	Foret de Breteuil	48.820	0.836	9	2
B057	Étang de L'Embranchoir	48.102	4.077	8	0
B058	Foret D'Othe	48.054	3.524	8	0
B059	Étang de Beine	47.824	3.745	6	0
B060	South of Chevillon	47.909	3.197	8	0
B061	East of Bellegarde	47.892	2.483	16	0
B062	Étang du Grand Vau	48.047	2.161	8	0
B063	Close to Chateau de Buglain	47.760	1.869	0	8
B064	Étang de Pescheux	47.588	1.156	0	8
B065	Étang des Greles – three individuals sequenced	47.493	0.438	3	18
B066	Lac de Rille, Langeais	47.328	0.418	0	8

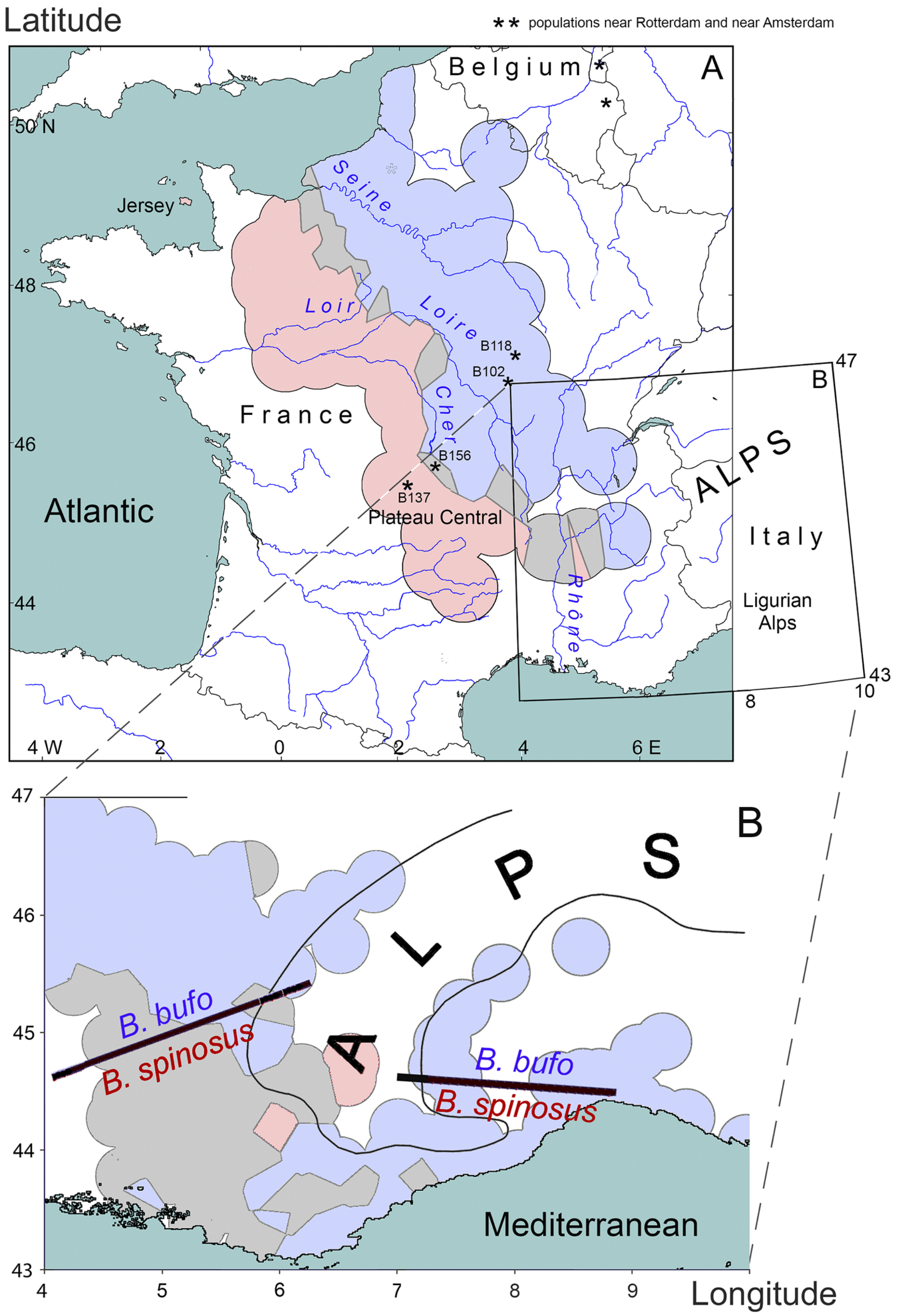
Table 1. (Continued.)

Population number	Locality – note	Northern latitude	Eastern longitude	mtDNA haplotype typical for	
				Bufo bufo	*Bufo spinosus*
B067	Roads between Continvoir, Avrillé-Les-Ponceaux, Les Essards and St-Michel-sur-Loire	47.376	0.269	0	19
B068	Northeast of Ville-aux-Clercs	47.929	1.111	0	8
B069	Folleville	49.018	0.206	1	12
B070	Étang de la Herse	48.404	0.551	0	19
B071	Villez-sur-le-Neubourg	49.159	0.863	8	0
B072	Lyons-la-Foret	49.406	1.487	8	0
B073	Mont Asselin	49.617	0.789	8	0
B074	La Vallée Becquerel	49.307	0.663	7	0
B075	Hardanges	48.354	0.399	0	9
B076	Northeastern edge of Foret de Pail	48.403	−0.213	0	8
B077	Northeast of St-Denis-d'Orques	48.052	−0.269	0	8
B078	Close to Bois de Brice	47.892	−0.285	0	8
B079	North of Juigne-sur-Sarthe	47.875	−0.283	0	8
B080	North of Chapelle-d'Aligné	47.748	−0.219	0	8
B081	Etang du Perré	47.526	−0.020	0	8
B082	Etang de Jumeaux	47.370	1.085	0	8
B083	North of Chapelle-Guillaume	48.128	0.903	0	8
B084	West of Bonneval	48.190	1.349	8	0
B085	Chalou-Moulineux	48.391	2.030	8	0
B086	Chanteau	47.963	1.977	8	0
B087	La Renardiere	47.899	1.657	1	12
B088	Between Dhuizon and La Ferte-St-Cyr	47.621	1.680	0	8
B089	South of Gy-en-Sologne	47.330	1.579	0	8
B090	Halfway between Nançay and A71-E09 motorway	47.344	2.112	0	8
B091	Halfway between Villegenon and Dampierre-en-Crot	47.445	2.591	5	7
B092	Northwest of Lavau	47.631	2.931	8	0
B093	St-Georges-sur-Moulon	47.187	2.440	2	11
B094	St-Georges-sur-Arnon	47.007	2.095	0	8
B095	Etang des Trois Biches	46.823	2.082	0	8
B096	Etang de St-Bonnet	46.649	2.693	8	0
B097 #	Etang de Miquet	46.301	2.949	8	0
B098 #	Foret Dominiale des Prieurés Bagnolet	46.661	3.185	8	0
B100 #	Sauvigny-les-Bois	46.969	3.286	8	0
B101 #	East of Devay	46.814	3.582	8	0
B102 #	Southeast of Chateau-Chinon	47.017	3.986	8	0
B103	Étang de Vauvillard	46.813	4.278	8	0
B104	Étang des Pionniers	46.577	4.016	8	0
B106	Bois de la Vallée	46.237	3.679	8	0
B108	Between Lafaurie and Jarjat	44.926	4.567	8	4
B110	Lone de l'Ove, South of Valence	44.871	4.850	7	5
B111	Southwest of Chabeuil	44.891	5.000	0	7
B112	Northeast of Barbieres	44.961	5.156	14	7
B115	Lac Mort	45.029	5.787	8	0
B116	Vergisson	46.308	4.723	8	0
B117	Bois des Vallottes	46.669	4.708	8	0
B118 #	Étang de Vernidard	47.352	4.104	8	0
B119 #	South of Vitry-Laché	47.185	3.570	8	0
B120	Southeast of Dun-sur-Auron	46.846	2.622	8	0
B121	South of Ste-Colombe-des-Bois	47.304	3.134	8	0
B122	South of Licheres-sur-Yonne	47.501	3.588	8	0

Table 1. (Continued.)

Population number	Locality – note	Northern latitude	Eastern longitude	mtDNA haplotype typical for	
				Bufo bufo	*Bufo spinosus*
B123	East of Cerdon	47.646	2.413	0	8
B124	St-Piat	48.561	1.585	8	0
B125	East of Pellerine	48.311	1.034	0	8
B126	Puy-Guillaume	45.965	3.496	8	0
B127	Croizet-sur-Gand	45.915	4.225	8	0
B129	Plan d'Eau des Lésines	45.962	5.583	8	0
B132	Lac de Malaguet	45.249	3.713	0	8
B133	SW of Trécisse	45.656	3.920	8	4
B134	Boussac	46.355	2.184	0	8
B135 #	Fontanieres	46.135	2.546	8	0
B136 #	Solignat	45.813	2.301	0	8
B137 #	Peyrelevade	45.701	2.038	0	8
B138	Jersey – several localities	49.2	−2.1	0	28
B139	Mortagne au Perche	48.572	0.595	3	19
B140	Foret du Perche et de la Trappe	48.600	0.639	6	14
B141	Beaulieu	48.679	0.746	22	18
B142	Chateau des Bois Fresnes/Center parks	48.719	0.832	6	4
B143	Bazoches sur Hoene, Montmean	48.543	0.508	3	5
B144	Pre en Pail	48.447	−0.201	0	8
B145	Mouettes	48.896	1.363	8	0
B146	Durtal	47.672	−0.265	0	8
B148	le Poitrineau, Saumur	47.281	−0.132	0	13
B149	Les quatre Vouges	48.800	1.016	8	0
B150 #	Beissat, near Magnat l'Etrange	45.776	2.286	0	6
B151	Roche	46.134	2.546	1	0
B152	les Pecheries, Mautes	45.930	2.412	0	1
B153	Lavaud Blanche, Auzances	45.996	2.466	0	1
B155 #	Etang de Basvilette, Crocq	45.877	2.370	0	8
B156 #	les Vergnes, Auzances – transect central locality	46.044	2.498	2	6
B158	Le Mas, Evaux les Bains	46.165	2.523	8	0
B159 #	Etang de Gandalogne, Viers, Magnat l'Etrange – southernmost locality in transect	45.781	2.297	0	8
B160	Jupilles, Fontaine de la Coudrie, Foret de Berce	47.795	0.372	0	8
B186	nr 142 Center parks	48.724	0.827	13	5
B187	La Houssaye	48.886	1.381	23	0
B188	Foret de Multonne, Mont des Avaloirs	48.443	−0.132	0	20
B360	Fontainebleau	48.356	2.709	5	0
B400	le Joinchet, St Laurent des Eaux	47.699	1.664	0	6
B402	Mazeirac, St Simon	44.950	2.488	0	8
B403	Sangayrac, St Amans des Cots	44.682	2.668	0	8
B404	la Planque, Nayrac	44.599	2.653	0	8
B405	le Cantabel, Severac le Chateau	44.297	3.040	0	8

Additional transect populations are: B171 Alexanderpolder, Rotterdam, the Netherlands (51.953 N, 4.542 E), B172-173 Amsterdamse Waterleiding Duinen, Zwarteveldkanaal and van der Vlietkanaal, the Netherlands (52.343 N, 4.554 E), B359 Boven Nieuwstadt, the Netherlands (51.040 N, 5.853 E), B464 Ventuille, Beaune-d'Allier nr. Montmarault (46.283 N, 2.927 E) and B472-476 Les Censes, Waimes and Rurhof, Elseborn, Belgium (50.47 N, 6.13-6.15 E). For population genetic data see table 2.


Latitude
** populations near Rotterdam and near Amsterdam
A
Belgium
50 N
48
46
44
Jersey
Seine
Loir
Loire
Cher
B118
B102
B156
B137
France
Plateau Central
Atlantic
Rhône
ALPS
Italy
Ligurian Alps
B
47
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B. bufo
B. spinosus
B. bufo
B. spinosus
Mediterranean
Longitude

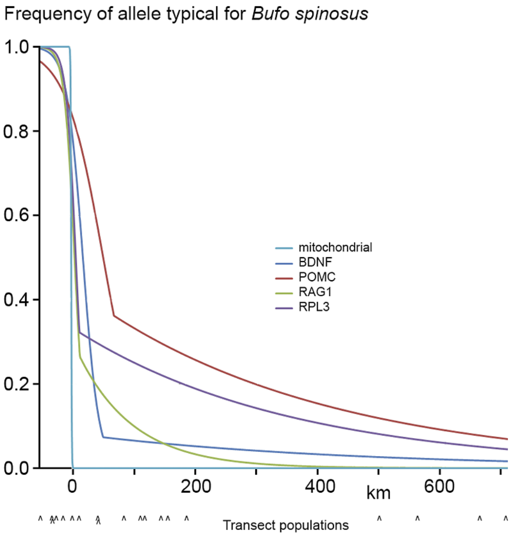

Figure 2. Variation in the frequency of 'spinosus' molecular genetic markers in a *Bufo spinosus* to *B. bufo* transect in central France estimated by geographical cline analysis. For transect location see fig. 1. The position of the studied populations is shown by small arrowheads. The zero position refers to the centre of the mitochondrial DNA cline, at population B156.

difficult to assess. However, data for this region and adjacent Italy have recently become available (Arntzen et al., 2017a) and are reproduced in fig. 1B. From the same source we plot the position of the *B. bufo*-*B. spinosus* contact zones at either side of the Alps, as determined from morphology and nuclear markers.

The species transition in central France is described by a steep cline in mitochondrial DNA and smoother clines for nuclear loci (fig. 2, the supporting data are in table 2). The central point for mtDNA is adjacent to population B156 at 46.044 N, 2.498 E, as this is the only population in the transect with mtDNA haplotypes of both species. Hence, the maximum width of the species transition is determined by 'pure' populations at either side. These are population B155 with 8 'spinosus' haplotypes and B135 with 8 'bufo' haplotypes. The distance between the populations is 29.4 km, which is at par with bounds of the 95% confidence intervals for the cline estimates at 0.1 km and 17.4 km. At ca. 61 km the nuclear clines are discordant, significantly wider than the mitochondrial cline (table 3). The cline centres are on average 21.1 km displaced towards the *B. bufo* side of the transect, significantly (not coincident) for BDNF and POMC and not so (coincident) for RAG1 and RPL3.

Discussion

We mapped *B. bufo* and *B. spinosus* from mtDNA over a large part of France to document their mutual range border and to complete the picture from the Atlantic to the Mediterranean. Species identification from morphology works well in the British Isles (Jersey) and in France, but not in Italy (table 4). As is to be expected, individuals of hybrid origin have intermediate phenotypes (Arntzen et al., 2016) and cannot (and should not) be allocated to either parental species. The mitochondrial and nuclear clines line up in north-western France and in central France, suggesting that the western part of the two-species range border is adequately documented. However, at southern latitudes the species-specific signatures are markedly dissimilar for nuclear versus mitochondrial DNA.

General features of the species contact zone are the absence of large areas of *B. bufo*-*B. spinosus* sympatry, its long, narrow and more

Figure 1. (A) Distribution of *Bufo bufo* and *B. spinosus* across France with the delineation of the species contact zone approximated from mitochondrial DNA haplotypes. Population samples have either *B. bufo* haplotypes (blue shading), *B. spinosus* haplotypes (red shading) or both (grey shading). The spatial extrapolation in Dirichlet cells does not exceed ca. 40 km. The position of the transect used for cline analyses is indicated by asterisks for the central and outermost populations B156, B137, B102 and B118. Four populations at the northern fringe of the transect are also shown. (B) Distribution of *B. bufo* and *B. spinosus* at the Mediterranean side of their joint distribution (from Arntzen et al., 2017a). Colour coding as in (A), and the spatial extrapolation of Dirichlet cells does not exceed ca. 25 km. The black bars represent *B. bufo*-*B. spinosus* hybrid zones west and east of the Alps.

Table 2. Molecular genetic data used for cline fitting over a transect in central France. Fs is the frequency of haplotypes and alleles typical for *Bufo spinosus*. *N* is the sample size.

Population	Distance from B156 (km)	mtDNA		BDNF		POMC		RAG1		RPL3	
		Fs	*N*	Fs	*N*	Fs	*N*	Fs	*N*	Fs	*N*
B137	−52.1	1.000	7	1.000	3	0.833	3	1.000	3	1.000	3
B150	−32.2	1.000	6	1.000	6	1.000	6	0.917	6	1.000	6
B159	−31.6	1.000	8	0.938	8	0.938	8	0.938	8	1.000	8
B136	−28.9	1.000	8	1.000	5	0.900	5	1.000	5	1.000	5
B155	−19.8	1.000	18	0.944	18	1.000	18	1.000	18	0.941	17
B156	0.0	0.545	11	0.727	11	0.750	10	0.700	10	0.850	10
B135	9.6	0.000	18	0.769	13	0.731	13	0.346	13	0.385	13
B464	42.7	0.000	11	0.000	10	0.650	10	0.200	10	0.500	2
B097	44.4	0.000	8	0.250	8	0.563	8	0.188	8	0.563	8
B098	84.2	0.000	8	0.071	7	0.429	7	0.143	7	0.143	7
B100	112.8	0.000	8	0.063	8	0.313	8	0.000	8	0.000	8
B101	118.7	0.000	8	0.000	8	0.375	8	0.125	8	0.000	8
B119	145.7	0.000	8	0.063	8	0.375	8	0.063	8	0.188	8
B102	157.0	0.000	8	0.143	7	0.286	7	0.143	7	0.571	7
B118	188.5	0.000	8	0.063	8	0.125	8	0.000	8	0.000	8
B472	504.1	0.000	28	0.000	28	0.089	28	0.000	28	0.204	27
B359	567.6	0.000	6	0.000	6	0.167	6	0.000	6	0.000	6
B171	669.2	0.000	7	0.125	8	0.000	7	0.000	7	0.000	7
B172	713.5	0.000	20	0.000	20	0.125	20	0.000	20	0.000	20

Table 3. Parameter estimates for the maximum-likelihood geographical clines shown in fig. 2. Position of the clines is relative to the one for mitochondrial DNA and cline width is 1/maximum slope. Distances are in km. Confidence intervals in parentheses are based upon the 2log-likelihood unit support limits. δ and τ are the shape parameters for tail fitting at the right side. The estimated frequencies at either end of the cline are unity for *Bufo spinosus* and zero for *B. bufo*. For a visualization of the nuclear clines with their confidence intervals and population data see online supplementary fig. S2.

Genetic marker	Model selection	Position (confidence interval)	Width (confidence interval)	Right tail	
				δ	τ
mtDNA	TypN	0 (−2.94-2.52)	1.82 (0.065-17.35)	None	None
BDNF	TypR	19.07 (8.50-29.45)	52.21 (31.51-82.98)	33.11	0.032
POMC	TypR	52.27 (17.31-111.48)	124.16 (65.53-302.33)	17.63	0.125
RAG1	TypR	5.48 (−3.83-16.57)	33.98 (20.08-67.20)	8.69	0.127
RPL3	TypR	7.49 (0.94-19.36)	31.74 (16.26-64.05)	5.90	0.033

or less linear aspect, with an overall geographical pattern similar to that of other species pairs, like *Triturus cristatus* and *T. marmoratus* (Arntzen and Wallis, 1991; Wielstra et al., 2014), or – not in France but more to the north and the east – *Natrix* grass snakes (Kindler et al., 2017). Some topographical correlates of the zone's position can tentatively be proposed. The zone starts at the mouth of the Seine and in central France its location appears to coincide with the rivers Loir, Loire and Cher. In north-western France the zone runs along the 'Collines de Normandie' low mountain range, and in Italy the zone is located at the northern slopes of the Ligurian Alps. However, the positioning of the contact zone does not seem affected by the Plateau Central and runs straight across the Rhône river into the Alps. Here the position of the hybrid zone partially coincides with the Isère river.

For *B. bufo* the upper limits in altitudinal range are around 2400 m a.s.l. (Sinsch et al., 2009) and because toad density declines with altitude, the French and Italian ranges of *B. bufo* are effectively separated by the Alps. These areas represent different intraspecific lineages

Table 4. Overview on the performance of morphological and molecular characters in use for the identification of *Bufo bufo* and *B. spinosus* in western Europe. Character states are diagnostic (plus), ambiguous (plus-minus), or not diagnostic (minus).

Character	British Isles	France			Italy
		Northwest	Centre	Southeast	
Morphology					
Body size	+	−	NS	+	−
Parotoids	+	+	NS	+	−
Metatarsus tubercle	+	+	NS	NS	NS
Mitochondrial DNA	+	+	+	−	−
Nuclear DNA – microsatellites	+	+	NS	NS	NS
Nuclear DNA – SNPs					
BDNF	+	+	+	+	+
POMC	+	+	+	+	+
RAG1	+	+	+	±	+
RPL3	+	+	+	+	±
Source	1-3	4-6	7	8	8

1 – Arntzen et al. (2014), 2 – Wilkinson et al. (2007), 3 – JWA unpublished data, 4 – Arntzen et al. (2013b), 5 – Arntzen et al. (2017b), 6 – Trujillo et al. (2017), 7 – present paper and 8 – Arntzen et al. (2017a). NS = not studied.

(Arntzen et al., 2017a). West of the Alps the mutual range border with *B. spinosus* is situated at ca. 45.0 N and east of the Alps at ca. 44.5 N (fig. 1). In the four transects studied to date, *B. bufo* and *B. spinosus* do exchange genetic material, i.e., they hybridize. At an average width of ca. 61 km at nuclear loci, the hybrid zone in central France appears wider than at the Atlantic side (53 km; Arntzen et al., 2016) and the Mediterranean side of France (9 km) and Italy (20 km; Arntzen et al., 2017a). Important features of this hybrid zone are its unimodal structure and the asymmetry in patterns of marker introgression, with *B. spinosus* haplotypes in the *B. bufo* side of the contact but not the other way round. This has been attributed to the existence of a moving hybrid zone, with *B. bufo* moving southwards at the expense of *B. spinosus*, resulting in introgression in the direction of the advancing species (Arntzen et al., 2017a).

If there are no ecological associations with hybrid zone location this would suggest that ecological constellations influence the mutual toad distribution differently across the species contact. Given the extensive interspecific hybridization observed over the four transects, in different ecological settings, the *B. bufo*-*B. spinosus* contact classifies as a heterogeneous hybrid zone (*sensu* Espregueira-Themudo et al., 2012). The European common toad hybrid zone offers good opportunity for genome-wide analyses in search of both consistently differentiated genomic regions across transects (candidate ‘barrier genes’) as well as locally adapted variants. The system is a promising model for the study of the genetic architecture of species differences. Particular assets are that the species are widespread, abundant and pond-breeding and that they have low dispersal.

Our results can be directly used to delineate species ranges, which is challenging in cases where morphology alone can lead to confusion or there is simply no available information to draw the line (e.g. Sillero et al., 2014). This is the case for these two species, which have an extinction risk status that is currently under re-assessment by the IUCN. While both species currently inhabit relatively large areas and are not considered to be in decline (Crochet et al., 2004; Arntzen et al., 2017b), population declines have also been reported (Petrovan and Schmidt, 2016). One initial step in this assessment will involve delimiting their respective ranges, for which we expect our results will be helpful.

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