



A new vertebrate species native to the British Isles: *Bufo spinosus* Daudin, 1803 in Jersey

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Recent molecular and morphological studies have shown that *Bufo bufo* and *B. spinosus* are genetically distinct and morphologically diagnosable across a relatively narrow contact zone in northern France and should be regarded as different species. However, the species identity of the neighbouring populations of *Bufo* on the British Channel Island of Jersey has not been investigated. We here present new molecular (a mtDNA RFLP assay plus sequences of the nuclear RAG1 gene) and morphological evidence that these populations are to be assigned to *B. spinosus*, and can thus be considered an addition to the native British herpetofauna. Jersey toad populations are declining and have a distinct breeding ecology compared to other populations in mainland Britain. We discuss the results in the light of amphibian conservation efforts in Jersey.

Key words: *Bufo bufo*, *B. spinosus*, Channel Islands, Jersey, morphometrics, mtDNA, nDNA, RAG1

INTRODUCTION

Species relationships within the common toad *Bufo* species group were recently elaborated by Recuero et al. (2012), who identified four distinct lineages within the range of *B. bufo* (Linnaeus, 1758) *sensu lato*. These corresponded to *B. bufo* (Linnaeus, 1758) from most of Europe including northern and eastern France into Russia (including toads from Great Britain, Scandinavia, Italy, the Balkans and the larger part of Turkey), *B. eichwaldii* Litvinchuk, Borkin, Skorinov and Rosanov, 2008 from the Talysh mountains of Azerbaijan and Iran (see also Litvinchuk et al., 2012), *B. spinosus* Daudin, 1803 from North Africa, Iberia and much of France, and *B. verrucosissimus* (Pallas, 1814) from the Caucasus and the north-eastern corner of Turkey. The sister species to *B. bufo sensu stricto* is not *B. spinosus* (which was until recently regarded as a subspecies of *B. bufo*) but the Caucasian *B. verrucosissimus* (Arntzen et al., 2013a). The ancestor of *B. spinosus* was isolated in Iberia (from the ancestor common to *B. bufo* and *B. verrucosissimus*) by the formation of the Pyrenees some 9 million years ago (Recuero et al., 2012). Most French and all Iberian and North African common toads should be referred to as *B. spinosus* based on the type locality of this taxon near Bordeaux in south-western France (Daudin, 1803), since that locality falls well within the

area occupied by the westernmost clade described by Recuero et al. (2012).

The spinous warts formerly considered typical of *B. spinosus* are *not* diagnostic for this species, but occur in specimens of both *B. bufo* and *B. spinosus*, particularly in more southerly parts of their respective ranges, and the two species are superficially similar. Arntzen et al. (2013b) described the contact zone between *B. spinosus* and *B. bufo* in France and revealed morphological character states that allow the species to be distinguished by non-molecular methods. In northern France, *B. bufo* and *B. spinosus* can be identified by the degree of posterior divergence of the parotoid glands and by the size and shape of the metatarsus tubercle. Parotoids are more divergent, as measured by parotoid angle, and the metatarsus tubercle is longer and narrower in *B. spinosus* than in *B. bufo* (see Figs. 2, 4 and 5 in Arntzen et al., 2013b).

The border of the distribution of *B. spinosus* in France and its contact zone with *B. bufo* is approximated by a line running from the city of Caen in the northwest to Lyon in the southeast, and by the river Rhone (Arntzen et al., 2013b; see Fig. 1). The distribution of *B. spinosus* (to the south and west of that line), therefore, encompasses at its northernmost edge the Contentin Peninsula in Normandy, France. The specific status of the toads on the British Channel Island of Jersey, located to the south-

west of the Contentin Peninsula, has hitherto not been established.

Jersey was the last of the Channel Islands to be separated from the European mainland with the final inundation of the English Channel between 9,000 and 6,000 years ago (Johnston, 1981). The island's common toads, until now assumed to be *B. bufo*, appear to have been declining for at least 40 years (Le Sueur, 1968, 1976; Buley, 1995; Beebee & Griffiths, 2000). They were once reportedly extremely common there; numbers being described as "scarcely credible" (see citations in Le Sueur, 1968). The conservation and ecology of toads in Jersey has been the subject of a PhD thesis (Wilkinson, 2007) and there has been some investigation of their conservation genetics (Wilkinson et al., 2007), whom also noted marked differences in the Jersey toads' reproductive ecology as compared to those in mainland Britain. Toads in Jersey begin breeding commonly in January and also make substantial use of, often very small, ornamental garden ponds for their reproduction, a trait not usually seen on the mainland. Additionally, the species has a cultural significance for Jersey Islanders who sometimes refer to themselves as "crapauds" (both French and Jèrriais for toads) and to the toads as "Jerseymen" (Le Sueur, 1968). Toads do not occur naturally on any of the other Channel Islands (Arnold, 1995). The assignment of Jersey toads to either *B. bufo* or *B. spinosus*, therefore, has implications for their conservation management as a potentially unique element of the British herpetofauna with its own distinct autecology.

METHODS

Morphological identification - Morphological characters, including snout-urostyle length (SUI), anterior and posterior parotoid distance (Pda and Pdp, respectively), length and width of the metatarsus tubercle (MTI

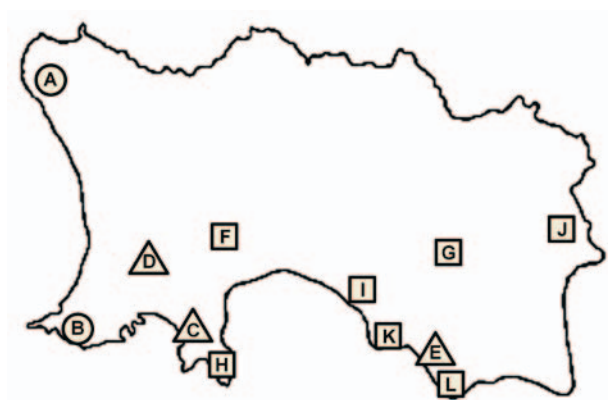


Fig. 1A. Sample locations on the island of Jersey. Locations reflect the main distribution of toads in the south and west of the island. (A) Canné de Squez, (B) Gorselands, (C) Ouaisné, (D) St. Brelade, (E) St. Clement 1, (F) Beaumont, (G) Grands Vaux, (H) Noirmont, (I) Bellozanne, (J) St. Martin, (K) St. Helier, (L) St. Clement 2. For locality coordinates see Appendix I. Circles - both morphological and molecular samples, triangles - morphological samples only, squares - molecular samples only.

and MTw, respectively) and parotoid angle (Pa) were measured during spring 2013 in 51 live Jersey toads as in Arntzen et al. (2013b), plus three Jersey and one 'Guernsey' specimens from the collection of the British Museum of Natural History (BMNH). Morphological and molecular sample locations in Jersey are shown in Fig. 1A. Metatarsus tubercle measurements were taken on the right side of the body. Parotoid angle was taken from digital photographs, parotoid divergence (Pd) was calculated as Pda/Pdp and metatarsus size and shape were calculated as MTI/SUI and MTw/MTI, respectively. Toads measured from life were released at the same place following measurement and photography. Toads from Jersey were compared with *B. bufo* from three localities in northern France (Audresselles, Autreppes and Sorques) and *B. spinosus* from two localities in western France (Jublains and Gizeux) (Fig. 1B), with data taken from Arntzen et al. (2013b). In order to investigate any absolute body size (SUI) differences between *B. bufo* and *B. spinosus*, available data for large samples were compared over a south to north transect across western Europe.

Data for bivariate data plots and logistic regression analysis were untransformed in order to obtain results that are directly interpretable and can be used in the field. For discriminant analysis, data were ln-transformed to increase statistical normality of the data and to reduce possible effects of allometric growth. The standardised

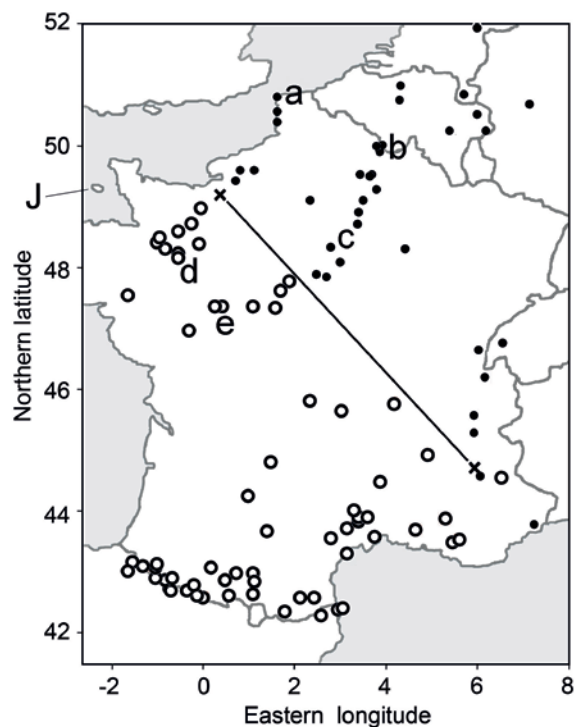


Fig. 1B. Distribution of *Bufo* toads across France and neighbouring regions, with solid round symbols for *B. bufo* to the northeast of the diagonal line and open round symbols for *B. spinosus* to the southwest of the diagonal line. Two localities shown with a cross have mtDNA haplotypes for both species. After Arntzen (2013b). Populations mentioned in the text are: a) Audresselles, b) Autreppes, c) Sorques, d)ublains, e) Gizeux and J) Jersey.

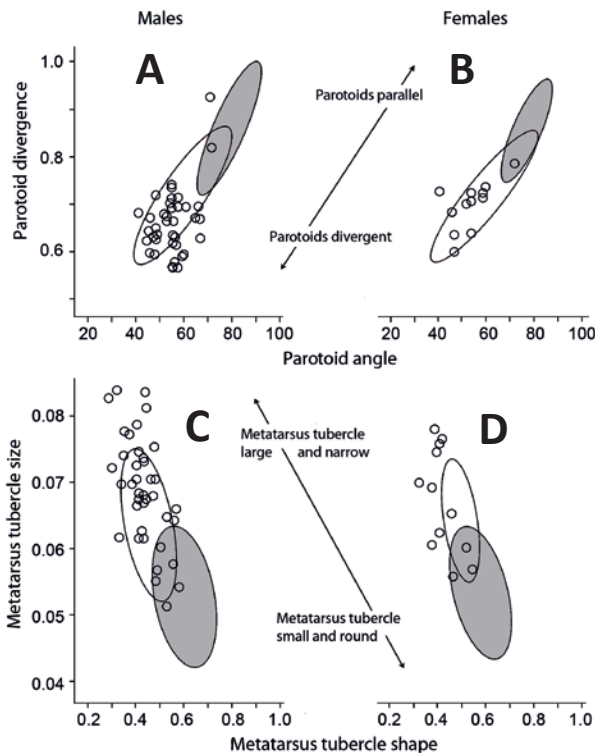


Fig. 2. Bivariate plot of parotoid (A, B) and metatarsus tubercle (C, D) character sets for male (A, C) and female (B, D) live toads from north-western France. Data are summarised by ellipses that represent the mean plus and minus one standard deviation in toads (*Bufo bufo* shaded and *B. spinosus* unshaded). Toads from Jersey are shown individually by open round symbols. Note that the bulk of the individual data points coincide with *B. spinosus* and not *B. bufo*, stronger so for the parotoid measurements than for the metatarsus tubercle measurements.

residuals were calculated for the linear regression of $\ln\langle\text{character}\rangle$ on $\ln\langle\text{SUL}\rangle$ to reduce the effect of overall size in the analysis. With discriminant analysis we calculated the probability for each of the newly measured toads from Jersey to associate with either *B. bufo* or *B. spinosus* from the continent. Statistical analyses were carried out with SPSS v.21 (SPSS, 2012) and confidence ellipses of the mean plus or minus one standard deviation in bivariate plots were determined with MyStat v.12 (MyStat, 2008).

Small amounts of tissue were obtained from Jersey toads as tadpole tail tips ($n=21$ from six localities) or samples from road-killed adult toads ($n=4$ from three localities); see Fig. 1A and Appendix 1. DNA extraction and mitochondrial DNA-based species assignment by RFLP analysis was carried out as described by Arntzen et al. (2013b). Additionally, a nuclear marker, RAG1, was amplified and sequenced in 4 samples from Jersey ($n=10$; Appendix 2) using primers Amp-RAG1F and Amp-RAG1-R1, with sequences and protocols described in San Mauro et al. (2004). For reference, additional specimens from the remainder of the distribution of both *B. bufo* ($n=59$) and *B. spinosus* ($n=45$) were also sequenced and included in subsequent analyses (see Appendix 2). A sequence of *Bufo gargarizans andrewsi* from GenBank (Accession Number: DQ158353, from Pramuk, 2006)

was used as an outgroup in phylogenetic analyses. Sequences were inspected and edited using Sequencher v.5.0.1 (Gene Codes Corp., Ann Arbor, MI, USA) and aligned in Mesquite (Maddison & Maddison, 2011). Two haplotypes per individual were phased using SeqPHASE (Flot, 2010) and PHASE v.2.1.1 (Stephens et al., 2001), with default settings. We tested for recombination with the phi-test implemented in SplitsTree v.4.13.1 (Huson & Bryant, 2006). Haplotype networks were constructed with HaploViewer (Available from: <http://www.cibiv.at/~greg/haploviewer>) using a maximum clade credibility consensus tree estimated by BEAST v.1.8 (Drummond et al., 2012), under a Yule coalescent prior. The substitution model K80+I was selected based on the Bayesian Information Criterion as implemented in jModeltest (Darriba et al., 2012). BEAST analyses were run for 20 million generations. Adequate mixing was assessed by inspection of the log file in Tracer v. 1.5 (Rambaut & Drummond, 2007) and was reached after a burn-in period of about 10% of the run length.

RESULTS

Morphological data observed in adult toads from Jersey strongly overlap with those recorded for *B. spinosus* from the continent (and not *B. bufo*) for both character sets that we studied (parotoids, metatarsus tubercle) for males as well as for females (Fig. 2). The logistic regression parameters of Arntzen et al. (2013b: Table 1a) classified the Jersey toads as *B. spinosus*, with a few exceptions depending on the character set used (see Table 1). Discriminant analyses of size-corrected data classified the far majority of Jersey toads as *B. spinosus* - 31 out of 39 (80%) for the male toads (Wilks' lambda 0.510, $df=4$, $p<0.0001$) and 10 out of 12 (83%) for the female toads (Wilks' lambda 0.663, $df=4$, $p<0.001$). In terms of absolute body size, data collected across a western Europe transect reveal a larger body size in *B. spinosus* than in *B. bufo*, regardless of latitude (Fig. 4). On this character, the toad populations studied from Jersey also classify as *B. spinosus*. The same holds true for the four preserved Channel Islands toads, all females, from the BMNH (Appendix 1). On account of the parotoid and metatarsus tubercle characters, the three Jersey toads classify as *B. spinosus*, whereas the only known specimen from Guernsey classifies as *B. bufo* (Fig. 3).

All 21 tadpoles and four adults from Jersey analysed in the mtDNA RFLP assay showed the characteristic restriction profile of *B. spinosus* (as in Arntzen et

Table 1. Number of male and female Jersey toads out of the total that are classified as *Bufo spinosus*, with the logistic regression formulae derived by Arntzen et al. (2013b), per character set.

Character set	Males	Females
Parotoids	38/39 (97%)	11/12 (92%)
Metatarsus tubercle	36/39 (92%)	10/12 (83%)
Both character sets	38/39 (97%)	12/12 (100%)

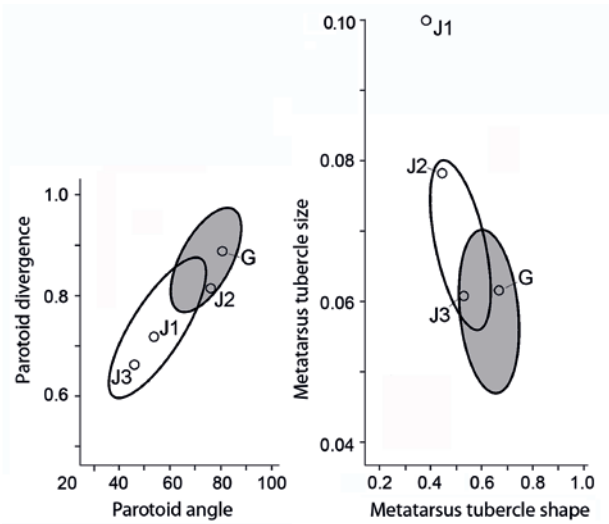


Fig. 3. As Fig. 2, females, with toads preserved in the collections of the museums of Leiden, The Netherlands; Paris, France and Madrid, Spain (data from Arntzen et al., 2013b). Three preserved toads from Jersey from the British Museum of Natural History, London, United Kingdom are shown individually: J1 - BMNH 1891.2.18.12, J2- BMNH 97.7.31.5 and J3 - BMNH96.10.6.4; G refers to BMNH 1909.12.14.25, reported from Guernsey where, however, no *Bufo* toads are currently known to occur (see text for details).

al., 2013b). With respect to RAG1, we obtained new sequences from 114 individuals (59 *B. bufo*, 55 *B. spinosus*) that were 876 bp long (GenBank Accession numbers: KJ544890–KJ544914). There was no evidence for recombination (phi-test, p -value=0.259). The 230 phased alleles were reduced to 26 unique haplotypes: 14 in *B. spinosus*, 11 in *B. bufo* and one in the outgroup. Figure 5 shows the haplotype network built with HaploViewer, with reference samples of *B. bufo* and *B. spinosus* forming two separate groups, shaded according to species assignment based on mtDNA profile. All samples from Jersey had the most common *B. spinosus* RAG1 alleles (haplotypes 1 and 2; see Fig. 5 and Appendix 2). Three individuals from the French populations of Sorques ($n=2$) and Autrepes ($n=1$) had mtDNA of *B. bufo* but a RAG1 haplotype commonly found in *B. spinosus* (Fig. 5).

DISCUSSION

Arntzen et al. (2013b) described the approximate ranges of *B. spinosus* and *B. bufo* in France (see Fig. 1B) and, through comparison of morphological and molecular data, identified the characters by which field identification is possible. The latter can be summarised as a relatively wide head, reflected by posterior parotoid divergence, and longer, narrower metatarsus tubercles in *B. spinosus* as compared to *B. bufo*. By the same criteria, both morphological and molecular data in the present study indicate that Jersey toads should be assigned to *B. spinosus*. Indeed, 97% and 100% of male and female Jersey toads respectively can be assigned to *B. spinosus*

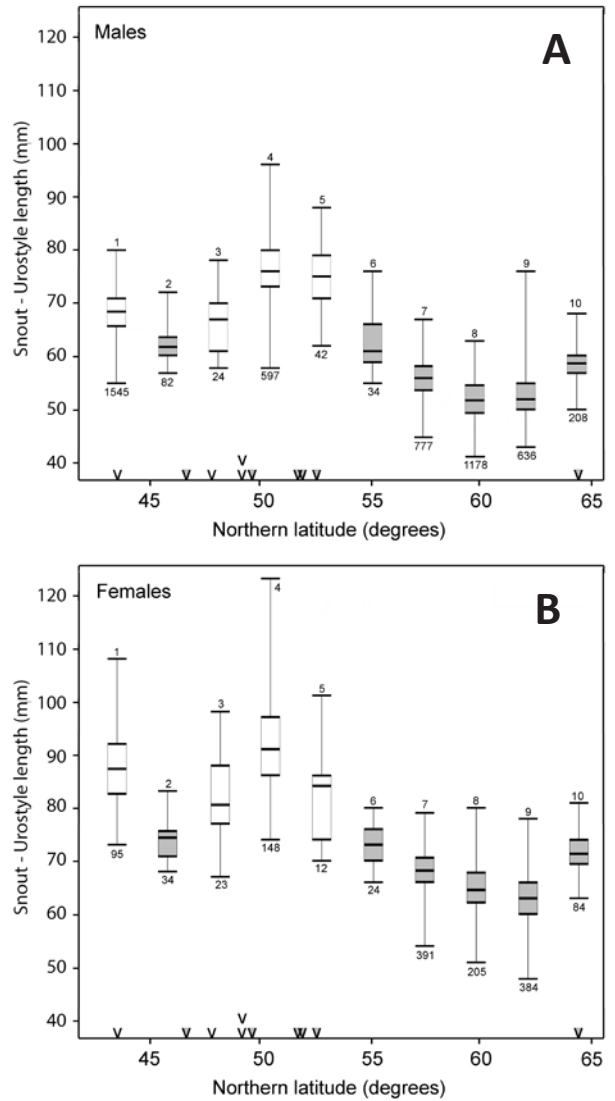


Fig. 4. Body sizes expressed in snout-urostyle length (SUI, in mm) of *Bufo* toads in a south to north transect across western Europe, for males (A) and females (B). Shown are population number, maximum SUI, second quartile, median, third quartile and minimum SUI, followed by sample size. Open bars refer to *B. spinosus* and filled bars refer to *B. bufo*. Note that *B. spinosus* are larger than *B. bufo*, irrespective of latitude. From left to right populations are: 1: southern France and 2: Switzerland (Hemelaar, 1988), 3: western France (Arntzen et al., 2013b), 4 and 5: Jersey (Wilkinson, 2007 and present study), 6: northern France (Arntzen et al., 2013b), 7: Germany and 8: The Netherlands (Hemelaar, 1988), 9: Colony Reservoir, Charnwood, Leicestershire, United Kingdom (JWA, unpublished data from 1992) and 10: Norway (Hemelaar, 1988). The precise latitude of the populations is indicated along the horizontal axis by open arrowheads for *B. spinosus* and filled ones for *B. bufo*.

without recourse to molecular data (Table 1).

The relatively large size of Jersey toads, as compared to those from mainland Britain, was noted by Smith (1951) and also observed in our data. Indeed, the SUI of *B. spinosus* is greater than that of *B. bufo* irrespective of latitude and adult males with SUI>65 mm and adult

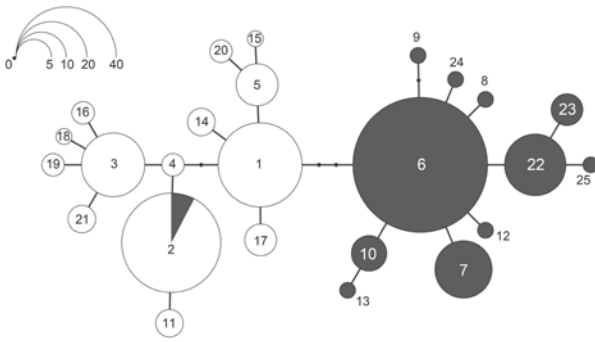


Fig. 5. Haplotype network of RAG1 sequences in *B. spinosus* (open circles) and *B. bufo* (solid circles). Circle sizes are proportional to haplotype frequency, as indicated in the scale. All Jersey samples have the most common *B. spinosus* alleles (haplotypes 1 and 2). The outgroup *B. gargarizans* has been removed for clarity. Note the presence of an allele typical for *B. spinosus* (haplotype 2) in three individuals of *B. bufo* as identified from morphology and mtDNA (see text for details).

females with $SUI > 75$ mm are mostly *B. spinosus* (Fig. 4). The observed size difference between mainland Britain and Jersey toads can now be attributed to the presence of an additional native species in the British Isles, without invoking the growth advantages often assumed to be conferred by a warmer climate (Arnold & Burton, 1978). The three toad morphotypes present and native within the British Isles therefore correspond to *B. bufo* in Great Britain and many offshore islands such as Anglesey and the Isle of Wight, *B. spinosus* exclusively in Jersey and *B. calamita* Laurenti 1768 (= *Epidalea calamita*) in some coastal and heathland areas of Great Britain. No toads are present in Northern Ireland. Species can be visually separated most easily by parotoid positioning (degree of posterior parotoid divergence, Fig. 6).

Four toads from the Channel Islands kept at the BMNH were all females with $SUI \geq 97$ mm. The sampling presumably reflects a collecting bias for a large body size considered to be unusual and indeed well outside the normal range for toads from mainland Britain (Fig. 4, see e.g., Smith, 1951). The specimen from Guernsey originates from historical introductions, now extirpated (pers. comm. the late Charles David, Guernsey Biological Records Centre; Arnold, 1995) which had been assumed to originate from Jersey. However, the positioning of the parotoids, and shape and relative size of the metatarsus tubercle are reminiscent of *B. bufo*, though this female was also of very large body size. A molecular genetic analysis to elucidate the geographical origin of this toad may be desirable, but falls outside the scope of the present study.

Molecular identification on the basis of mtDNA and nDNA attributed all Jersey material unequivocally to *B. spinosus*. However, three individuals (5%) from the French populations of Sorques ($n=2$) and Autrepes ($n=1$) had mtDNA (and the morphology) of *B. bufo* but one RAG1 haplotype commonly found in *B. spinosus* (Fig. 5). This observation is in line with the hypothesis that continental *B. spinosus* previously had a wider distribution

and that interprets the “*spinosus* alleles” in the current *B. bufo* range as a “genetic footprint”, reflecting the past presence of the donor species (Arntzen et al., 2013b).

Mainland British *B. bufo* are typical explosive breeders, spawning usually during one or two weeks in March or April (Smith, 1951; Beebee & Griffiths, 2000; Inns, 2009). Wilkinson (2007), however, noted that the first Jersey spawn appears commonly in January (exceptionally in December) and that breeding can extend over many weeks if interrupted by periods of cold weather. This temporal pattern has also been observed in the department of Sarthe, France in 2013, with *B. spinosus* spawning earlier than *B. bufo* – the species’ breeding activities were separated by an unusual cold spell in the second half of March (JWA, pers. obs). Toads from around the type locality of *B. spinosus* in Aquitaine, France, breed between January and March (Cistude Nature, 2010), and also relatively early elsewhere in the south and west of the country (see e.g., Hemelaar, 1988; Lesbarrères et al., 2010). Amplexus and spawning of *B. spinosus* can be observed as early as December in southern Spain (González de la Vega, 1988; Díaz-Paniagua et al., 2005) and Portugal (L. Russell, pers. comm.), though most spawning occurs in January or early February. Toad spawn in lowland areas of Iberia generally appears very early in the season, relative to other amphibians (Campeny & Montori, 1988; Richter-Boix et al., 2006). The latter authors showed a negative correlation between toad breeding and temperature in north-east Spain (i.e. they bred just after the coldest part of the year, as soon as temperatures began to rise), consistent with the pattern seen in Jersey. Though January temperatures in Jersey (minima and maxima) are

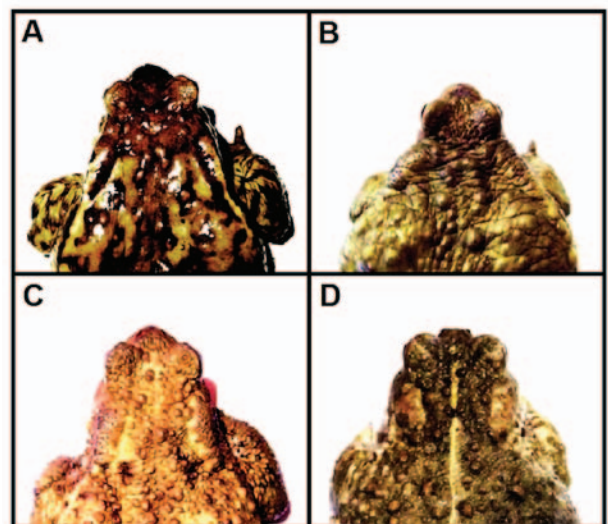


Fig. 6. Comparison of parotoid positioning in three species of European toad. (A) *Bufo spinosus* from Ouaisné, Jersey (78 mm SUI), (B) *Bufo spinosus* from Loupfougères, Northwest France (80 mm SUI), both showing marked posterior parotoid divergence, (C) *Bufo bufo* from Woolmer, UK (69 mm SUI) showing near-parallel (only slightly divergent) parotoids and, for comparison, (D) *Bufo (Epidalea) calamita* from Hengistbury Head, UK (68 mm SUI), showing posterior parotoid convergence. All specimens shown are male.

extremely variable (see <http://www.jerseymet.gov.je>), the onset of breeding of *B. spinosus* there corresponds well to an increase in the 40-day-mean temperature found by Reading (2003) to predict the onset of toad breeding in southern England (see also Wilkinson, 2007).

Several authors (Richter-Boix et al., 2006; Da Fonseca et al., 2008; Gómez-Rodríguez et al., 2009) report the dependence of *B. spinosus* on permanent ponds and, though these are present in Jersey in the form of man-made reservoirs, they are only irregularly used for toad breeding there. This may be due to the sometimes poor and variable quality of Jersey's groundwaters (Wilkinson, 2007). In contrast, Jersey toads are dependent on some 200+ garden or other ornamental ponds, mainly in the south of the island, and around 10 semi-natural ponds found on heaths and in sand-dunes in the west. All such ponds tend to dry out in the warm summers of Jersey, a factor possibly influencing the early appearance of toadspawn on the island. We hypothesise that the innate ability of *B. spinosus* to breed very early in the year, as evidenced by other lowland populations in France and Iberia, has proven adaptive for Jersey toads in conditions where permanent water bodies are naturally rare and available breeding sites are prone to drying.

Jersey *B. spinosus* have been isolated from French conspecifics for at least 6,000–9,000 years since the island was separated from the European mainland (Johnston, 1981), possibly rather longer (Wilkinson et al., 2007). Toads were formerly more widespread (Le Sueur, 1976), breeding in the small agricultural reservoirs which were once common. These are now often abandoned in favour of artificial water troughs inaccessible to breeding toads. Jersey populations are therefore increasingly isolated from one-another, showing high levels of genetic differentiation for relatively close sites (<1 km apart), with values occasionally in excess of those found between Jersey and French localities (Wilkinson et al., 2007). The absence of substantive gene flow among local populations of Jersey toad suggested by these data emphasises the need for toad conservation on the island, especially in the knowledge that *B. spinosus* is a unique element of the British herpetofauna. The situation is unlikely to improve naturally at present – the Jersey landscape is increasingly fragmented by development (new housing and associated roads) and water abstraction has increased to service new residences. Though new housing brings with it a proportion of properties with suitable ornamental ponds, colonisation by toads is often difficult and many garden populations rely on just one or two breeding females in any year (Wilkinson, 2007; Wilkinson et al., 2007), rendering garden populations susceptible to extirpation.

Herpetofauna conservation in Jersey is well-prioritised and amphibian species' recovery there has received a good deal of attention. The island's rarest amphibian, *Rana dalmatina*, once reduced to one breeding site (Baker & Gibson, 1995), has already benefitted from effective head-starting programmes (see Racca 2003; 2004; JARG, 2010; Wilkinson & Buckley, 2010). The Jersey toad has become another focus of efforts to maintain the island's biodiversity and will gain from the creation

of planned new breeding ponds (N. Cornish, pers. comm.) and monitoring through *Jersey Toadwatch* (see Wilkinson, 2007). The revelation of the Jersey crapaud as *Bufo spinosus*, with an autecology both distinct from mainland British toads and subtly different from continental conspecifics, will hopefully both (i) inform tailored actions for its conservation and (ii) maintain an emphasis on conservation of the island's herpetofauna.

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APPENDICES

Appendix 1. Data overview on adult morphology and tissue samples collected on the British Channel Islands toads. Codes are: m=male, f=female, ttt=tadpole tail tip voucher numbers, HF=material collected by Hugh Forshaw. For a map of Jersey localities A–L see Fig. 1A.

Field collected data: A – Canné de Squez, 49.249183 N, 2.250067 E; adult morphometrics 21m, 8f, adult body size 329m, 120f (Wilkinson, 2007), mtDNA ttt 1145, 1146, 1148. B – Gorselands, 49.176768 N, 2.229086 E, adult morphometrics 1m, 1f, adult body size 26m, 10f

(Wilkinson, 2007), mtDNAtt 1120-1122, 1124, 1125. C – Ouaisné, 49.178794 N, 2.181388 E, adult morphometrics 8m, 2f. D – St. Brelade, 49.191767 N, 2.209689 E, adult morphometrics 9m, adult body size 242m, 18f (Wilkinson, 2007). E – St. Clement 1, 49.173245 N, 2.082619 E, adult morphometrics 1m, 1f. F – Beaumont, 49.200847 N, 2.161691 E, mtDNAtt 1114, 1115, 1119. G – Grands Vaux, 49.204588 N, 2.084263. mtDNAtt 1126, 1127. H – Noirmont, 49.169942 N, 2.169633 E, mtDNA carcass adult 1150 (HF). I – Bellozanne, 49.249183 N, 2.250067 E, mtDNA carcass adult 1151, 1153 (HF). J – St. Martin, 49.207438 N, 2.038098 E, mtDNA carcass adult 1152 (HF). K – St. Helier, 49.178415 N, 2.104095 E, mtDNAtt 1139, 1140, 1142. L – St. Clement 2, 49.168290 N, 2.081542 E, mtDNAtt 1132, 1133, 1135-1137.

Material from the British Museum of Natural History, London, UK:1891.2.18.12 from Jersey, leg. G.A. Boulenger, SUI 104 mm; 96.10.6.4 from Jersey, leg. J. Hornell Esq, SUI 98 mm; 97.7.31.5 from Jersey, leg. Boulenger, SUI 98 mm; and 1909.12.14.25 from Guernsey, leg. Ms Power, SUI 97 mm. Notes: All females. Specimen 1891.2.18.12 has a markedly 'oblique' parotoid shape (sensu Arnold & Burton, 1978). It is of pale colour, presumably due from exposure to light in the exhibit. Specimen 1909.12.14.25 has an oddly shaped inner metatarsus tubercle at its right foot and we measured at the left foot instead.

Appendix 2. Material studied for the nuclear RAG1 gene in *B. bufo* and *B. spinosus*, with country, locality, coordinates and sample indication. RAG1 haplotypes are shown in **bold** type and correspond to those in Fig. 5.

France - Audresselles, 50.821140 N, 1.602310 E; 1a: **6, 7**; 2a: **6**; 3a: **6, 7**; 4a: **6**; 198: **6, 7**; 199: **6**; 200: **6, 7**; 202: **6**; Autrepes, 40.91492 N, 3.84678 E; 1: **6, 7**; 2: **8, 9**; 3: **6, 7**; 4: **7**; 162: **2, 6**; 174: **6, 7**; 175: **6**; 176: **6**; 177: **6**; 178: **6**; Gizeux, 47.37608 N, 0.26945 E; 211: **1, 11**; 212: **1, 2**; 213: **1**; Jublains, 48.23955 N, 0.55190 W; 7a: **1, 2**; 34: **1**; 35: **1, 11**; 68: **1, 11**; 69: **2**; 70: **1, 2**; 73: **2** and Sorques, 48.34485 N, 2.77790 E; 5: **2, 12**; 6: **6**; 7: **7**; 8: **6, 7**; 184: **6**; 185: **6**; 186: **6, 7**; 188: **6**; 189: **2, 6**.

Great Britain - Jersey, 49.200847 N, 2.161691 E; 1114: **2**; 1115: **2**; 1119: **2**; Jersey, 49.176768 N, 2.229086 E;

1120: **2**; 1121: **2**; 1124: **1, 2**; 1125: **2**; Jersey, 49.204588 N, 2.084263 E; 1126: **2**; 1127: **2** and Jersey, 49.169942 N, 2.169633 E; 1150: **1, 2**.

Greece - Dimitrios, 40.086617 N, 22.212417 E; IMS4744: **6**.

Italy - Canale Monterano, 42.140467 N, 12.097071 E; OMON2: **6**; OMON5: **6**; OMON7: **6**; Fiume Irminio, 36.928892 N, 14.673619 E; ORAG3: **22**; ORAG5: **22**; ORAG8: **22**; ORAG9: **22**; ORAG10: **22**; Gambarie, 38.180996 N, 15.845921 E; OGAM1: **6**; OGAM2: **22**; OGAM3: **6, 22**; OGAM21: **24, 25**; Lago dell' Antigola, 38.739557 N, 16.235975 E; OANG1: **22**; OANG2: **6, 23**; OANG3: **6, 23**; OANG4: **6, 23**; OANG5: **6, 23** and Monghidoro, 44.248303 N, 11.346230 E; OGH1: **6**; OGH2: **6**; OGH3: **6**; OGH5: **6**.

Portugal - Buçaco, 40.375670 N, 8.366310 W; IMS1437: **3**; IMS1438: **5, 16**; IMS1439: **5, 18**; Ermidas do Sado, 38.007028 N, 8.433750 W; IMS2902: **3**; IMS2903: **1** and Portalegre, 39.331300 N, 7.323281 W; PORTALEGRE: **3, 5**.

Serbia - Djurinci, 44.511867 N, 20.631867 E; IMS4736: **6**; IMS4738: **6**; Guberevac, 43.865000 N, 20.771150 E; IMS4746: **6**; IMS4747: **10**; Krepoling, 44.391750 N, 21.983483 E; IMS4763: **6**; IMS4764: **6**; IMS4765: **6**; Promaja, Vlasina Lake, 42.678183 N, 22.357183 E; IMS4732: **6**; IMS4733: **10** and Zlidul, 42.421883 N, 22.451667 E; IMS4735: **10, 13**.

Spain - 35 km NE Capileira, 36.973944 N, 3.322583 W; CAPI1: **3**; CAPI2: **1, 5**; CAPI3: **1, 5**; CAPI4: **1, 3**; Canillas de Aceituno, 36.870611 N, 4.080167 W; IMS1167: **1, 14**; IMS1168: **15, 16**; Fanlo-Escalona, 42.587611 N, 0.013139 W; BBU09: **1, 2**; BBU10: **1, 2**; Guadalupe, 39.517556 N, 5.348028 W; IMS3839: **2, 14**; IMS3840: **1, 14**; IMS3841: **19, 20**; Hecho, 42.736417 N, 0.760389 W; BBU01: **1, 2**; BBU02: **1, 3**; BBU03: **1, 2**; Laguna Grande de Gredos, 40.253056 N, 5.275833 W; IMS4256: **1, 2**; IMS4257: **1, 4**; IMS4258: **3, 21**; IMS4261: **3, 5**; Laújar, 37.085167 N, 2.904722 W; LAUJAR1: **3, 21**; LAUJAR2: **3, 21**; Marbella, 36.516660 N, 4.981680 W; IMS3813: **19, 20**; Ordesa-San Nicolás de Bujaruelo, 42.663361 N, 0.112556 W; BBU06: **1, 4**; BBU07: **3, 5**; BBU08: **3** and Sadernes, 42.278694 N, 2.590167 E; BBU11: **2**; BBU12: **2**; BBU13: **1, 2**.

Tunisia - Beni M'Tir, 36.737028 N, 8.717694 E; IMS1288: **17** and Feija, 36.497528 N, 8.305333 E; IMS1353: **17**.