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ORIGINAL ARTICLE

Inferring the degree of incipient speciation in secondary contact zones of closely related lineages of Palearctic green toads (*Bufo viridis* subgroup)

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Reproductive isolation between lineages is expected to accumulate with divergence time, but the time taken to speciate may strongly vary between different groups of organisms. In anuran amphibians, laboratory crosses can still produce viable hybrid offspring >20 My after separation, but the speed of speciation in closely related anuran lineages under natural conditions is poorly studied. Palearctic green toads (*Bufo viridis* subgroup) offer an excellent system to address this question, comprising several lineages that arose at different times and form secondary contact zones. Using mitochondrial and nuclear markers, we previously demonstrated that in Sicily, *B. siculus* and *B. balearicus* developed advanced reproductive isolation after Plio-Pleistocene divergence (2.6 My, 3.3–1.9), with limited historic mtDNA introgression, scarce nuclear admixture, but low, if any, current gene flow. Here, we study genetic interactions between younger lineages of early Pleistocene divergence (1.9 My, 2.5–1.3) in northeastern Italy (*B. balearicus*, *B. viridis*). We find significantly more, asymmetric nuclear and wider, differential mtDNA introgression. The population structure seems to be molded by geographic distance and barriers (rivers), much more than by intrinsic genomic incompatibilities. These differences of hybridization between zones may be partly explained by differences in the duration of previous isolation. Scattered research on other anurans suggests that wide hybrid zones with strong introgression may develop when secondary contacts occur <2 My after divergence, whereas narrower zones with restricted gene flow form when divergence exceeds 3 My. Our study strengthens support for this rule of thumb by comparing lineages with different divergence times within the same radiation.
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INTRODUCTION

A central assumption in evolutionary biology is that reproductive isolation accumulates with increasing genetic distance, 'more likely as a series of small steps than in a single genetic revolution' (Barton and Charlesworth, 1984), and thus will correlate with divergence time. However, comparative research in natural systems often neglects the time taken to speciate. In fact, diverging evolutionary lineages can be observed anywhere in the continuum from near-panmixia to various levels of increasing genetic isolation and distance, up to complete reproductive isolation. The evolutionary processes combine subtle to complex genomic changes through intrinsic mutation and drift (non-adaptive, 'neutral'), and/or selection caused by extrinsic pressures (adaptive, 'selective'; for example, Pereira and Wake, 2009), with ecology playing a role in most (Sobel *et al.*, 2010).

Hybridization has complex effects on the speciation processes as recently reviewed by Abbott *et al.* (2013). One fascinating aspect for evolutionary research is the formation of secondary hybrid zones, in which 'it is uncertain if barriers to gene flow will be strengthened or broken down due to recombination and gene flow' (Abbott *et al.*, 2013). While theory and empirical evidence suggest the latter is more likely, strongly selected genomic regions might pose exceptions (Abbott *et al.*, 2013). Despite the potential stochastic occurrence of single large-effect factors (*cf.* Barton and Charlesworth, 1984), generally a gradual increase in reproductive isolation can be expected over evolutionary time in allopatry. When such lineages come early into secondary contact, with few if any 'barrier loci' (Abbott *et al.*, 2013) evolved, gene flow may negate any incipient speciation (for example, Seehausen *et al.*, 2008). In more advanced stages of allopatric speciation, gradual build up of many isolating factors of small effect (for example, Barton and Charlesworth, 1984 and Abbott *et al.*, 2013) and necessary associations among such loci (Smadja and Butlin, 2011) can further contribute to reduce gene flow between diverging gene pools (Abbott *et al.*, 2013), so that the involved genomes become less permeable and no longer merge.

Considering the likely gradual build up of reproductive isolation (for example, up to a certain 'threshold above which observed differentiation is significantly greater than expected by neutral evolution alone', Nosil and Feder, 2012), we suggest that in closely related lineages with known divergence times (estimated from paleogeographic scenarios or molecularly dated), the degree of natural hybridization at secondary contacts may serve to better understand the timing of onset and progression of speciation. Several amphibians, particularly anurans, offer suitable species systems to test this

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Table 1 Localities, regions of origin, geographic coordinates (degrees) and number of green toad samples from larvae, subadults and adults (Supplementary Table S1 for further details)

					Adult							*Genetic diversity*	
Loc. number	*Province*	*Name of locality*	*Latitude (N)*	*Longitude (E)*	*Male*	*Female*	*Unsexed*	*Juvenile*	*Larvae*	*Age unknown*	*Total*	*A.r.*	H_e
1	Treviso	Nervesa della Battaglia	45.8494	12.1489	—	—	—	7	—	—	**7**	3.4	0.53
2	Treviso	Cusignana Bassa	45.7836	12.1942	—	—	3	1	7	7	**18**	3.3	0.53
3	Treviso	Montebelluna	45.7578	12.0524	5	—	2	5	6	—	**18**	3.9	0.59
4	Padova	Onara	45.6308	11.8262	—	—	—	3	—	5	**8**	3.8	0.63
5	Venezia	Jesolo	45.5457	12.6493	—	—	—	1	—	—	**1**	—	—
6	Venezia	Favaro Veneto	45.5236	12.2902	2	—	—	—	—	—	**2**	—	—
7	Venezia	Salzano	45.5306	12.1335	—	—	—	—	7	—	**7**	3.1	0.57
8	Venezia	Mestre	45.4991	12.2615	—	1	—	—	5	—	**6**	—	—
9	Venezia	Venezia	45.4807	12.3006	—	—	—	1	—	—	**1**	—	—
10	Venezia	Porto Marghera	45.4482	12.2349	—	—	2	—	—	—	**2**	—	—
11	Venezia	Treporti	45.4661	12.4550	—	—	—	—	3	—	**3**	—	—
12	Venezia	Island of S. Erasmo	45.4596	12.4208	—	1	4	1	—	—	**6**	—	—
13	Venezia	Venezia	45.4289	12.3600	1	5	—	—	8	—	**14**	—	—
14	Venezia	island of Lido di Venezia	45.4260	12.3881	—	—	1	—	1	—	**2**	—	—
15	Venezia	island of Lido di Venezia	45.3420	12.3237	—	—	—	—	4	—	**4**	—	—
16	Venezia	Sambruson	45.4013	12.1003	—	1	—	—	—	—	**1**	—	—
17	Vicenza	Vicenza/Rettorgole	45.5660	11.5250	—	—	6	4	7	—	**17**	3.9	0.62
18	Padova	Abano Terme	45.3793	11.8049	—	—	—	—	3	—	**3**	—	—
19	Padova	Legnaro	45.3410	11.9629	—	—	—	—	—	2	**2**	—	—
20	Padova	Rosara di Codevigo	45.2887	12.0997	—	—	—	4	—	—	**4**	—	—
21	Padova	Arzergrande	45.2706	12.0658	—	—	—	—	—	1	**1**	—	—
22	Padova	Brugine	45.2810	11.9950	—	—	—	—	—	1	**1**	—	—
23	Venezia	Island of Pellestrina	45.2973	12.3092	1	—	—	7	—	—	**8**	3.5	0.61
24	Vicenza	Albettone	45.3605	11.6036	—	—	4	—	—	—	**4**	—	—
25	Verona	Caldiero	45.4167	11.1500	—	—	1	—	—	—	**1**	—	—
26	Vicenza	Spessa	45.3436	11.4433	—	—	—	—	—	9	**9**	3.5	0.58
27	Vicenza	Campiglia dei Berici	45.3222	11.5339	—	—	—	—	—	1	**1**	—	—
28	Verona	Cologna Veneta	45.3113	11.3855	—	—	—	—	—	10	**10**	3.2	0.6
29	Padova	Santa Croce	45.2544	11.5806	—	—	—	—	3	—	**3**	—	—
30	Padova	Monselice	45.2380	11.7263	—	—	12	—	11	—	**23**	3.9	0.64
31	Padova	Monselice	45.2234	11.7723	—	—	1	4	—	—	**5**	—	0.65
32	Padova	Megliadino San Fidenzo/Santa Margherita d'Adige	45.2236	11.5219	—	—	7	1	3	2	**13**	—	—
33	Padova	Piacenza d'Adige	45.1228	11.5286	—	—	3	—	3	2	**8**	3.7	0.64

Table 1 (Continued)

					Adult							*Genetic diversity*	
Loc. number	*Province*	*Name of locality*	*Latitude (N)*	*Longitude (E)*	*Male*	*Female*	*Unsexed*	*Juvenile*	*Larvae*	*Age unknown*	*Total*	*A.r.*	H_e
34	Venezia	Isola Verde	45.1681	12.3264	—	—	2	—	—	—	**2**	—	—
35	Venezia	Sant'Anna di Chioggia	45.1208	12.2614	—	—	2	2	—	—	**4**	—	—
36	Rovigo	Norge Polesine	45.0659	12.2262	—	—	1	—	—	—	**1**	—	—
37	Rovigo	Rosolina Mare	45.0565	12.2738	—	1	4	—	—	—	**5**	3.8	0.59
38	Rovigo	Scanarello	45.0138	12.3922	—	—	1	—	—	—	**1**	—	—
39	Rovigo	Ca' Venier	44.9590	12.3364	—	—	—	1	—	—	**1**	—	—
40	Rovigo	Adria	45.0238	12.0868	1	—	—	4	—	—	**5**	—	0.5
41	Rovigo	Baricetta	45.0610	11.9923	—	—	1	—	—	—	**1**	—	—
42	Rovigo	Pezzoli	45.0448	11.9316	—	—	1	—	—	—	**1**	—	—
43	Rovigo	Rovigo	45.0787	11.7799	1	—	4	8	6	—	**19**	3.7	0.63
44	Rovigo	Sant'Apollinare	45.0402	11.8230	—	—	1	—	—	—	**1**	—	—
45	Rovigo	Crespino	44.9988	11.8495	1	—	—	—	—	—	**1**	—	—
46	Rovigo	Fratta Polesine	45.0039	11.6453	—	—	1	—	—	—	**1**	—	—
47	Verona	Isola della Scala	45.2436	11.0117	—	—	7	—	—	—	**7**	4.2	0.7
48	Verona	Cerea	45.1927	11.2127	—	—	—	—	—	3	**3**	—	—
49	Mantova	Ostiglia	45.0733	11.1234	—	—	—	—	—	1	**1**	—	—
50	Mantova	Suzzara	45.0419	10.6942	—	—	—	—	—	14	**14**	3.5	0.54
51	Mantova	San Benedetto Po	45.0358	10.8793	—	—	—	—	—	1	**1**	—	—
52	Mantova	Pegognaga	44.9878	10.8696	—	—	—	—	—	5	**5**	2.5	0.44
53	Ferrara	Francolino	44.9018	11.6610	—	—	4	—	—	—	**4**	—	—
54	Ferrara	Ferrara	44.8269	11.6092	—	—	4	—	2	—	**6**	—	0.56
55	Bologna	Oasi Ca' la Riza	44.6675	11.4483	—	—	4	—	—	—	**4**	—	—
56	Modena	Campogalliano	44.6975	10.8456	—	—	—	—	5	—	**5**	2.3	0.4
57	Ravenna	Casalborsetti	44.5539	12.2783	—	—	—	—	1	—	**1**	—	—
58	Ravenna	Bagnacavallo	44.4292	11.9714	—	—	—	—	3	—	**3**	—	—
59	Ravenna	Russi	44.4122	12.0317	—	—	1	—	—	—	**1**	—	—
60	Ravenna	Campiano	44.2976	12.1956	—	—	—	—	1	—	**1**	—	—
61	Milano	Milano	45.4300	9.1700	—	—	2	—	—	—	**2**	—	—
62	Forlì—Cesena	Ronco	44.2028	12.0789	—	—	—	—	1	—	**1**	—	—
63	Rovigo	Rosolina Mare	45.1349	12.3235	—	—	1	—	—	—	**1**	—	—
				Total	**12**	**9**	**87**	**54**	**90**	**64**	**316**		

For populations where $n \geqslant 5$ individuals, the mean allelic richness (A.r.; based on a sample of five individuals) and observed heterozygosity (H_e) are provided.

assumption, but studies on incipient speciation in this group have rarely been performed.

Meta-analyses of anuran (bufonid) breeding experiments suggest that reproductive isolation (measured by reduction in hatching success, number of larvae produced, and percentage of metamorphosis) increases with phylogenetic distance (Malone and Fontenot, 2008). It appears that very large time spans (>8 My) are required to achieve hybrid infertility or inviability. We recently discussed such laboratory data on anurans (Colliard *et al.*, 2010), with divergence time estimates from immunological (Wilson *et al.*, 1974), allozyme (Sasa *et al.*, 1998) and mitochondrial distances (Malone and Fontenot, 2008), or mitochondrial and nuclear sequence data (Sumida *et al.*, 2007), showing that in the laboratory some anurans may still produce viable F_1 offspring after >20 My divergence, but may develop 'partial or complete hybrid inviability' after >8 My divergence (Sumida *et al.*, 2007). However, under natural conditions, several empirical studies on anurans suggest reproductive isolation to have arisen after divergence initiated in the Pliocene (5.3–2.6 My) or earlier, as best studied in parapatric *Bombina* (for example, Szymura, 1993, Kruuk *et al.*, 1999, Vines *et al.*, 2003 and Hofman *et al.*, 2007), assumed to have diverged between Upper Miocene and Lower Pliocene (*B. bombina–B. variegata*, 8.96 (12.74–4.93) My or 6.48 (8.89–4.19) My, depending on two different calibration settings; Pabijan *et al.*, 2013). Similar time frames were estimated for single hybrid systems of Australian and European hylid frogs (Hoskin *et al.*, 2005; Verardi *et al.*, 2009), South-American dendrobatids (Simões *et al.*, 2012) and green toads (Colliard *et al.*, 2010). By contrast, case studies on clades of more recent, Pleistocene (2.5 My–11 Ky) divergence (for example, 1.33 My in ranid frogs, Canestrelli and Nascetti, 2008; 1.69–0.33 My in bufonid toads, Sequeira *et al.*, 2011) may form 'wide hybrid zone(s) with a considerable genetic exchange between both gene pools' (Santucci *et al.*, 1996), where reproductive barriers could be weak or absent.

One challenge for testing an inverse relationship between divergence time and natural hybridization in anuran amphibians in the long term is the disconnected evidence from multiple (often) distantly related species groups. The radiation of Palearctic green toads (*Bufo viridis* subgroup) offers an excellent opportunity as it includes several lineages that arose at different times and form secondary contact zones. Specifically, the central and northeastern Mediterranean shores, the Apennine Peninsula and Sicily, are inhabited by four lineages with secondary contacts bringing together pairs with three different Plio-Pleistocene divergence times (Stöck *et al.*, 2006, 2008a, b). These are *Bufo variabilis* (Pallas, 1769) on the eastern Balkan Peninsula, in Asia Minor, and northern Central Asia, *B. viridis* (Laurenti 1768), on the western Balkan Peninsula and in Central Europe, *B. balearicus* (Boettger, 1880) on the Apennine Peninsula, Corsica, Sardinia and the Balearic Islands, and finally *B. siculus* Stöck, Sicilia, Belfiore, Buckley, Lo Brutto, Lo Valvo, Arculeo 2008, in Sicily.

We have recently shown that the Sicilian endemic *B. siculus* and the Italian mainland-origin *B. balearicus*, with an estimated divergence time of 2.7 My (4.9–1.1, Stöck *et al.*, 2008a), form a narrow hybrid zone east of Mt. Etna. Analysis of nuclear genomes showed very sharp transition at the contact, with essentially no admixture (Colliard *et al.*, 2010). The highest pairwise F_{ST} values (>0.50) were found between populations from each side of this contact zone (only ~16 km apart). All individuals from these populations were assigned with maximum likelihood (100%) to either *B. siculus* or *B. balearicus*, respectively. MtDNA analyses evidenced some limited bidirectional introgression over ~40 km, with few cases of cyto-nuclear discordances, testifying to rare events of past hybridization, but no wild-caught F_1 individuals. Altogether, this analysis suggested very low, if any, current gene flow, as furthermore supported by several experimental crosses, showing strong hybrid breakdown in F_2 and backcrosses (Colliard *et al.*, 2010).

In the present paper, we compare signatures of secondary contact and introgression in Sicily with another contact zone in northeastern Italy, where the same lineage *B. balearicus* meets another but more recently diverged lineage, namely *B. viridis*. As we show, this contact zone offers a striking contrast to the Sicily situation, with much wider introgression both at nuclear and at mitochondrial levels, suggesting that gene flow is barely constrained by intrinsic genomic incompatibilities in these less differentiated lineages.

MATERIALS AND METHODS

Sampling

Samples (buccal swabs from adults and subadults; muscle from road kills and scientific vouchers; tail tips from tadpoles) were collected during fieldwork (2008–2010; Figure 1, Table 1; Supplementary Table S1), or came from scientific collections (Museo di Storia Naturale di Venezia, MSNVE; Museo Civico di Storia Naturale di Ferrara, MCSNFE). Overall, 316 specimens from 63 localities were available across the eastern Po Plain, between the northern Apennine and the Venetian Pre-Alps. The majority of samples per population (term used in the sense of 'locality sample' throughout this paper) came from single sampling sites. As long as no potential barriers like rivers (see Discussion) or highways (for example, Forman *et al.*, 2003) were in between, very few samples from neighboring sites were pooled (Supplementary Table S1) if their distance was <2 km, corresponding to the lower limit of the migration distance of green toads in a single year (2–10 km; Blab *et al.*, 1991). This seems justified because the Po Plain is otherwise almost free of elevation differences and major natural barriers for amphibian movement. For tadpoles, to avoid the collection of siblings, samples were taken from unconnected ponds or, in rare instances, where it was not possible, very distant positions within the same pond, and in such cases always from differently sized cohorts.

DNA extraction and generation of genotype and sequence data

DNA was extracted using the Qiagen DNeasy kit. Twelve microsatellites, polymorphic in both species, were amplified for 254 samples as described (Colliard *et al.*, 2010, Dufresnes *et al.*, 2011). In 287 individuals, we sequenced *ca.* 860 bp of the mitochondrial *D-loop*, according to Stöck *et al.* (2006). For direct comparisons, only a fragment (591 bp) of this, also available from a previous study (Stöck *et al.*, 2008a), could be included to estimate the divergence time. We also cloned and sequenced 580 bp of a sufficiently variable nuclear intron of *alpha-Tropomyosin* (details: Stöck *et al.*, 2008a), in 13 individuals from the contact zone and representatives of both species from throughout their distribution ranges.

Sequence alignment and phylogenetic analyses

Sequences were edited in Sequencher v. 4.9 (Gene Codes) and aligned using Seaview v.4.2.4 (Gouy *et al.*, 2010). For the population analyses of mtDNA, we used the program TCS v.1.21 (Clement *et al.*, 2000). Phylogenetic analyses were made using PhyML, v.2.4.5 as implemented in Seaview (Gouy *et al.*, 2010), and HKY+G models (JModeltest v.0.1.1; Posada, 2008) for both mtDNA and nuDNA. We choose a BioNJ as a starting tree, and used the combined subtree pruning and regrafting plus nearest neighbor interchange options for tree improvement; otherwise, default parameters were used (http://atgc.lirmm.fr/phyml/ for details). We generated bootstrap values based on 1000 resampled data sets.

Molecular dating

Molecular dating for major mitochondrial and nuclear lineages was performed using BEAST v.1.6.1 (Drummond *et al.*, 2007; http://beast.bio.ed.ac.uk/). To obtain relative divergence time estimates for the most recent common ancestor of *B. viridis* vs *B. balearicus*, we calibrated the previously obtained divergence date for *B. siculus* vs *B. boulengeri* at 1.8 My (3.5–0.63, 95% highest posterior density intervals, HPDIs; Stöck *et al.*, 2008a), and the divergence of lineages

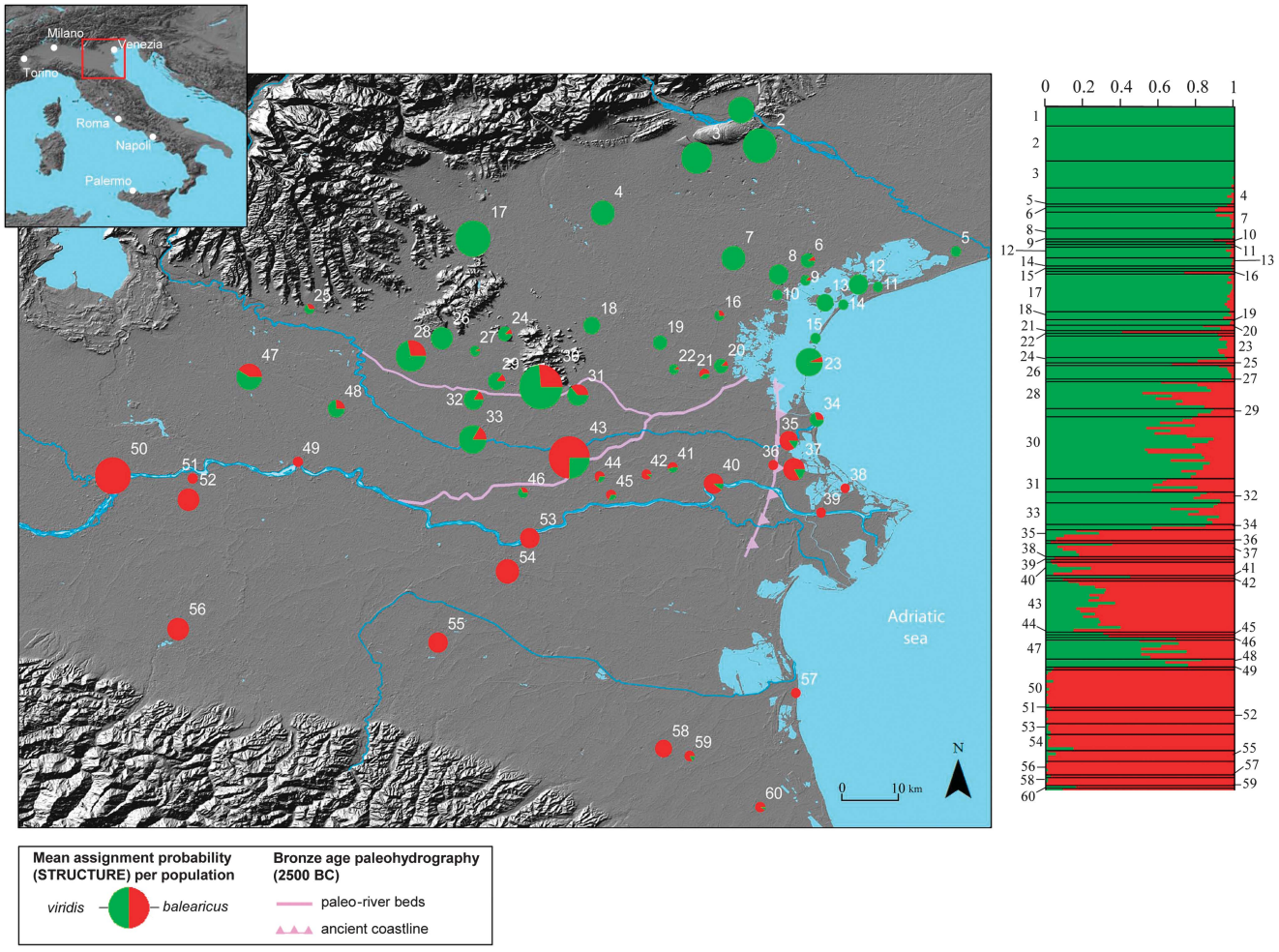

Figure 1 Assignment to *Bufo balearicus* or *B. viridis* for all populations, based on Bayesian clustering (Structure) of 12 microsatellite genotypes. Assignment per population (pie charts) corresponds to the average assignment probabilities of individuals at this location to each of the two groups. Pie size proportional to sample size; bar plots from Structure to the right ($K=2$); approximate Bronze Age paleohydrographic system (Piovan *et al.*, 2010) in purple.

boulengeri-siculus vs *balearicus-viridis* at 2.75 My (4.9–1.18) on a previously used fragment of the mtDNA control region (591 bp; Stöck *et al.*, 2008a), assuming an uncorrelated lognormal relaxed molecular clock and a Yule birthrate tree prior (constant speciation rate per lineage) as most appropriate for species level divergences (Drummond *et al.*, 2007). We applied the same calibrations for the nuclear data set (*alpha-Tropomyosin*). We used an UPGMA starting tree. Two independent analyses for 50 million generations were run with tree sampling every 1000 generations. Convergence and stationarity were checked in the program Tracer v.1.5. Results were combined in the BEAST module LogCombiner v.1.6.0. The 'burn-in' value was selected after visualizing log likelihoods associated with the posterior distributions of trees in Tracer. All trees generated before the log likelihood curve flattened out were discarded.

Demographic analyses

To calculate the distributions of observed and expected pairwise nucleotide site differences between individual mtDNA haplotypes under a model of demographic expansion (mismatch distributions), we used DnaSP v.5 (Librado and Rozas, 2009). We included only *D-loop* markers for which 874 bp 100% readable sequences were available. Ages of expansions were calculated from the parameter τ, estimated by DnaSP ($\tau = 2\,\mu t$, where μ is the substitution rate and t is the time since expansion), using a substitution rate for the *D-loop* of *ca.* 2% per million years (Stöck *et al.*, 2008a). In addition, we computed (DnaSP) the following tests of selective neutrality: Fu's F_s, Tajima's D and Ramos-Onsins & Rozas's R^2 (Ramos-Onsins and Rozas, 2002 and references therein); significances were tested by coalescent simulations (10 000 replicates).

Genotype data analyses

We used Micro-Checker v.2.2.3 (Van Oosterhout *et al.*, 2004) to exclude genotyping errors due to null alleles, stuttering and allelic dropout. Using Fstat v.2.9.3 (Goudet, 1995), we tested for linkage disequilibrium between each pair of loci in each population. Hardy–Weinberg equilibrium and pairwise differentiation (F_{ST}) were assessed for populations with sufficient sample sizes ($n \geqslant 5$) in Arlequin v.3.5 (Excoffier *et al.*, 2005). For these populations, we also computed allelic richness (A. r.) and observed heterozygosity (H_e) with Fstat, which performs a rarefication procedure to a common sample per locus. Analyses of microsatellite genotypes provided no evidence for large allelic dropout from any locus or population.

We used the Bayesian clustering algorithm Structure v.2.3 (Pritchard *et al.*, 2000) to assess interspecific and intraspecific genetic structures of populations based on microsatellite genotypes. We applied the admixture model and allowed for correlated allele frequencies between populations, as recommended for cases of subtle population structure. A range of different cluster sizes (K) from 1 to the number of localities per analysis was tested. Each run was replicated 10 times with 10^5 iterations following a 'burn-in' period of 10^4. To infer the number of clusters (K) that best fitted our data, we applied the ΔK *ad hoc* statistics (Evanno *et al.*, 2005).

In interspecific analyses, individuals were considered as hybrids if their Structure assignment probability to either cluster ($K=2$) was <0.9, with 90% credible intervals (CIs) neither reaching 0 nor 1, or if they were assigned by Structure to one clade (that is, nuclearly 'pure': 90% CI within 0.9 and 1) but contained the mitochondrial haplotype of the other lineage (cyto-nuclear

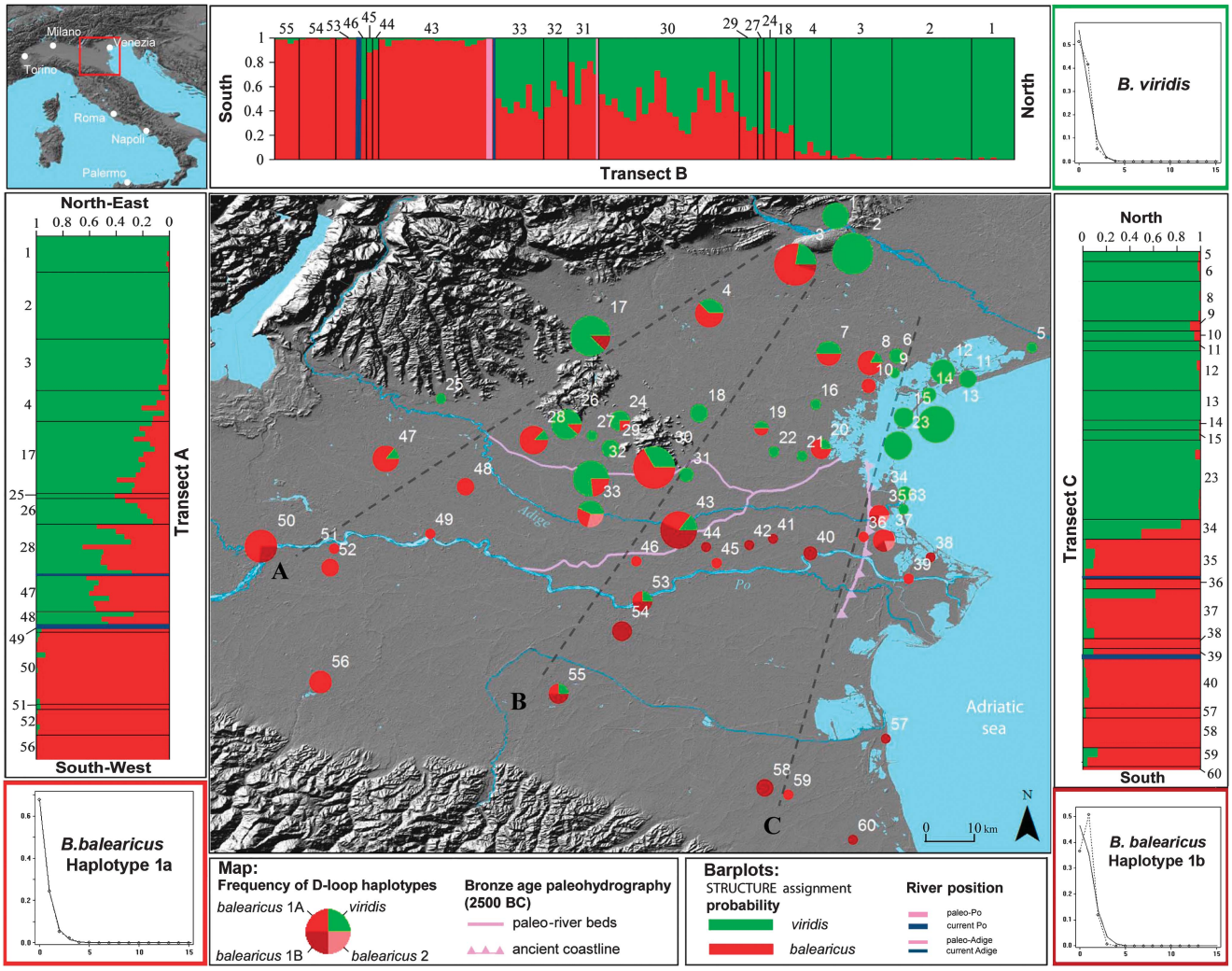

Figure 2 Frequency of mitochondrial *D-loop* haplotypes for all populations (map), mismatch distributions of three mtDNA haplotype groups (corners), and microsatellite genotypes (STRUCTURE assignment probabilities) along three transects A, B and C (barplots). Pie size is proportional to mtDNA sample size; approximate Bronze Age paleohydrographic system (rivers, coast line; Piovan *et al.*, 2010) in purple; relative locations of the modern Po and Adige Rivers in blue (in purple for paleo-rivers) within bar plots; mismatch distributions: the dotted line shows the frequency distribution of the observed pairwise differences; the solid line shows the frequency distribution of the expected pairwise differences under the sudden expansion model.

Table 2 Neutrality tests (mitochondrial DNA) for the *B. viridis* clade, and the *B. balearicus* subclades 1A and 1B

	n	*Fu's* F_s	*Tajima's D*	*Ramos-Onsins & Rozas's* R^2
Bufo viridis	129	−6.8***	−1.7***	0.35*
Bufo balearicus 1A	97	−11.9***	−2.3***	0.34*
Bufo balearicus 1B	33	−3.1**	−1.4*	0.08*

*P-value < 0.1, **P-value < 0.05, ***P-value < 0.001.

discordance). Considering CIs allows distinguishing between individuals that harbor alleles uninformative about the source taxa, and confidently assigned hybrid or 'pure' individuals (for example, Sá-Pinto *et al.*, 2010). To get insights into the nature of hybrids, we analyzed our microsatellite data set with NEWHYBRIDS v.1.1 (Anderson and Thompson, 2002), which computes the Bayesian posterior probability of assignment of each individual to several genotypic classes (parental, F_1, F_2, backcrosses). Runs were repeated with various numbers of iterations. Several individuals were pre-assigned as 'parents' (using the *z* parameter), but we restricted this pre-assignment to individuals whose assignment by STRUCTURE was higher than 0.99 (with 90% confidence interval within 0.9 and 1), to discard potential highly backcrossed individuals. We also conducted a principal component analysis using PCAGEN v.2 (Goudet, 1999) on allelic frequencies to visualize population differentiation. Significance of axes was tested by 10 000 randomizations of genotypes.

Geographic transects

We conducted additional STRUCTURE analyses along three transects evenly distributed in the flat corridor delimited by the Pre-Alps in the northwest and Adriatic Sea in the east, crossing the Po and Adige rivers (Figure 2; A–C), and presumably corresponding to major, topographically possible migration directions of the two toad lineages (transect A: locs. 1–4, 17, 25–26, 28, 47–52, 56; transect B: locs. 1–4, 18, 24, 27, 29–33, 43–46, 53–55; transect C: 5, 6, 8–15, 23, 34–40, 57–60). Along transects, the frequencies of alleles diagnostic for *B. balearicus* (that is, absent from pure *viridis* populations) were calculated for localities with $n \geqslant 4$ (transect A: locs. 1–4, 17, 26, 28, 47, 50, 56; transect B: locs. 1–4, 30, 31, 43, 53–55; transect C: 5, 6, 8–15, 23, 34–40, 57–60). For two transects (A, B; C was dismissed due to a sampling gap in its southern part), we computed genetic clines for the mitochondrial marker and for microsatellites possessing species-specific alleles (that is, alleles that contributed to

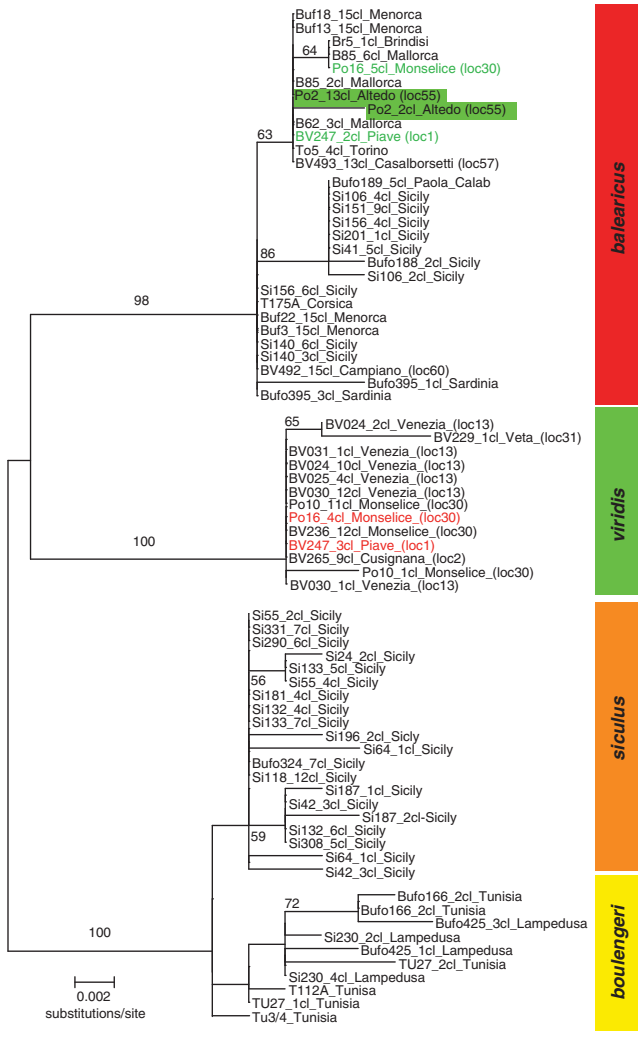

Figure 3 Phylogenetic tree based on a nuclear sequence marker. Maximum likelihood tree based on clones ('cl') obtained from 580 bp of an intron of *alpha-Tropomyosin*. Sample number (sometimes several with same haplotype and locality) is followed by locality information and population number (as in Figure 1, Supplementary Table S1); individual 'Po2', highlighted green, possessed a *D-loop* of one lineage but *Tropomyosin* alleles from the opposite species; toads BV247 and Po16 each contained an allele from *balearicus* and *viridis*, respectively.

cluster differences and for which frequencies, when narrowed down to a two-allele system, were higher than 0.9 in pure populations of one species). We further cross-checked the diagnostic value of alleles according to coordinates on the first axis of a multiple correspondence analysis (Genetix v.4.05; Belkhir *et al.*, 1998) of allelic frequencies of pure populations (Gay *et al.*, 2008). For each microsatellite, selected alleles were assigned to species-specific compound alleles to reduce the variation to a two-allele system. Along each transect, clines were fitted to allelic (microsatellites) and haplotype (mtDNA) frequencies with the program Cfit v.7.0 (Gay *et al.*, 2008), using a three-part stepped cline model, comprising a central sigmoid and two exponential tails (Szymura and Barton, 1986). We performed a likelihood search for a common center (coincidence) and slope (concordance) of all clines (Gay *et al.*, 2008), reiterating each fit with different random seeds to check for convergence. Models with different constraints (common center, common slope and both) were successively fitted to all markers simultaneously, and likelihood-ratio tests were performed to compare constrained with unconstrained clines. In the final models, individual clines, for which the constrained models were rejected (likelihood-ratio tests significant, 5% level), were fitted independently. For each transect, we chose the final model with the lowest Akaike information criterion (AICc).

To examine isolation by distance and the role of major rivers (Adige, Po) as potential barriers to dispersal, we computed partial correlations between pairwise F_{ST}, geographic distances and a matrix of the number of rivers between populations ($n \geqslant 5$) by partial Mantel tests (Arlequin). The geographic distance matrix was obtained using Geographic Distance Matrix Generator v.1.2.3 (Ersts, 2006). Significance of correlations was tested by a permutation procedure (10 000 permutations).

RESULTS

Phylogenetic analyses and divergence times

The *viridis* mtDNA clade is homogenous across the study region, and reveals a significant range expansion that occurred *ca.* 16 Ky ago (Figure 2; Table 2). In contrast, the *balearicus* haplotypes form three subclades (1a, 1b and 2; Figure 2; Supplementary Figure S1) with some geographic structure. Two subclades show significant signs of expansions (Figure 2; Table 2), dating back to 43 Ky ago for haplotype 1A (the most northern), but only 3 Ky for haplotype 1B (Figure 2). Transects show a large region of co-occurrence and a smooth transition in frequencies over >100 km from *balearicus* to *viridis* haplotypes. Networks for the major clades are provided with maximum-likelihood bootstrap support in Supplementary Figure S1 (see also trees in Stöck *et al.*, 2006, 2008a).

Nuclear sequences (*alpha-Tropomyosin* intron) also differentiate two highly supported clades, corresponding to *B. viridis* and *B. balearicus*, respectively (Figure 3). The *B. balearicus* clade is widespread on the Apennine Peninsula, from the Po Plain (study area) to southern Italy, and also found on Sardinia, the Balearic Islands and easternmost Sicily. Interestingly, two individuals (Po16, loc. 30; BV247, loc. 1, Figure 3) harbored an allele from *balearicus* as well as *viridis*, demonstrating their nuclear hybridity.

Molecular dating of mitochondrial and nuclear sequence data using BEAST suggests a Lower Pleistocene divergence of *B. viridis* and *B. balearicus*. Estimates point to 1.9 My (95% HPDI: 2.5–1.3 My) for the mtDNA *D-loop*, and 2.0 My (95% HPDI 3.04–1.09 My) for intronic sequences of the nuclear *Tropomyosin*. The posterior predictions for the divergence time between *B. siculus* and *B. balearicus* were very close to the mode assumed for the prior, and consistent between mtDNA and nuDNA: namely 2.65 (95% HPDI: 3.3–1.9 My) and 2.5 My (95% HPDI: 3.5–1.55 My) for the *D-loop* and the *Tropomyosin*, respectively.

Population structure

In two populations, assigned to *B. balearicus* (pop. 50, 56), null alleles were detected for locus C201 and corrections performed. We did not find significant linkage disequilibrium in any population after sequential Bonferroni corrections. All but one population (pop. 40, *B. balearicus*) met Hardy–Weinberg expectations. Analyses of microsatellite genotypes using Structure clearly grouped individuals into two clusters ($K = 2$, best fitting the whole data set), corresponding to *viridis* and *balearicus* gene pools (Figure 1). Most individuals from the northeastern part of the study area (north of Euganei hills and Venice; pop. 1–4, 7–8, 12–13, 15) were assigned to pure *viridis*, whereas individuals from south of the Po River were mainly assigned to pure *balearicus* (pop. 50, 52, 54–56, 58). Individuals from localities in between showed intermediate but robust probabilities of assignment (that is, 90% CI neither reaching 0 nor 1), suggesting clear signs of nuclear admixture (Figure 1).

Pairwise F_{ST} values revealed a clear pattern of isolation by distance, as illustrated by the principal component analysis (Figure 4). The first

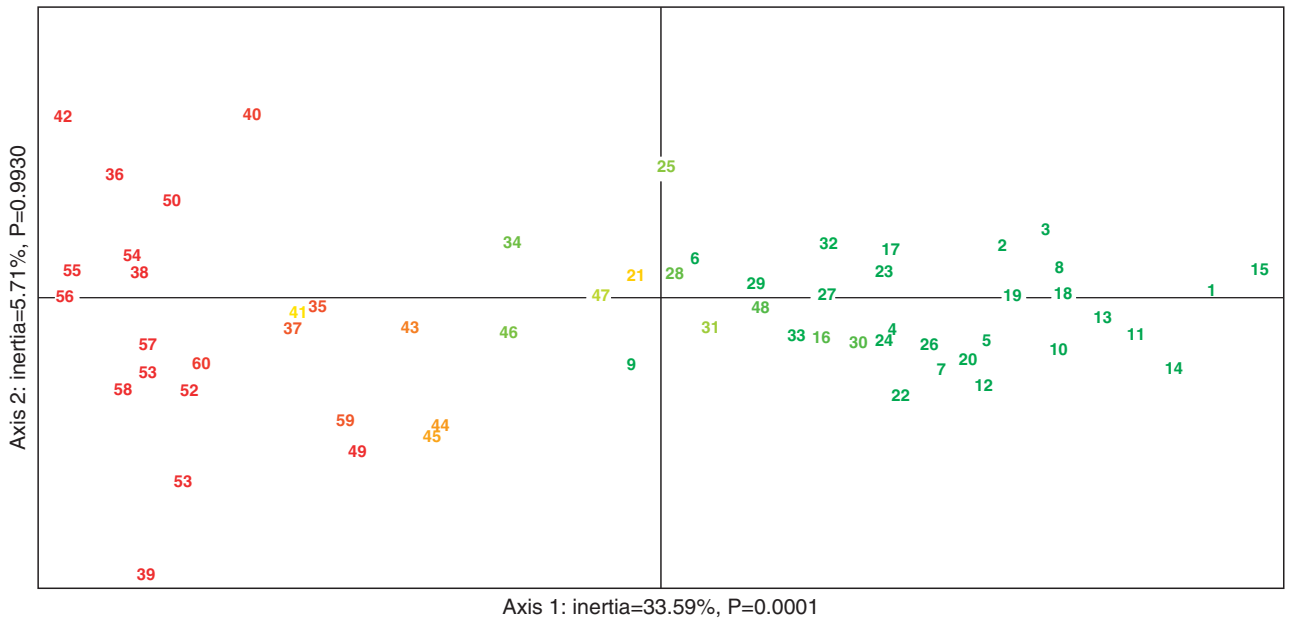

Figure 4 Principal component analysis on allelic frequencies including all populations, using PCAGEN (Goudet, 1999). Only the first axis is significant ($P<0.01$); green/red coloration is proportional to average assignment probability to *B. viridis/B. balearicus*, respectively (see Figure 1).

Table 3 Partial correlations between F_{ST}, geographic distances and number of large rivers between populations, analyzed by partial Mantel tests

	F_{ST} *vs distance + current rivers*	F_{ST} *vs distance + paleo-rivers*	F_{ST} *vs distance*
Correlation F_{ST} −distance	0.67***	0.67***	0.67***
Correlation F_{ST} −rivers	0.72***	0.66***	—
Determination of F_{ST} by distance	25%	31%	44%
Determination of F_{ST} by rivers	36%	29%	—
Explained variance of F_{ST}	61%	60%	44%
Unexplained variance of F_{ST}	39%	40%	56%

Distinct tests considered the current major rivers or the paleo-river beds (Bronze Age, ca. 5 Kya, Piovan *et al*., 2010).

and only significant axis explaining 33.6% of the variance ($P=0.0001$, bootstrapping) opposes pure *viridis* (right) to pure *balearicus* (left) populations. The transition in-between is continuous through the contact zone. The populations farthest away from the contact zone center display the highest F_{ST} values (0.53), whereas those at the contact zone show little or no differentiation (F_{ST} 0.00–0.03; Supplementary Table S2).

Isolation by distance was confirmed by Mantel tests; over the whole data set, pairwise F_{ST} increased significantly with geographic distance (44% of the variance explained, $P<0.001$). In addition, partial Mantel tests revealed a significant effect of major rivers (Po and Adige). Including the present-day riverbeds explained 61% of the variance, of which 25% was due to geographic distance, and the remaining (36%) to the number of large rivers (0, 1 or 2) separating populations. Interestingly, very close and significant values are also obtained when historical (Bronze Age), rather than present-day riverbeds are included (Table 3).

Genetic diversity was quite uniform over the study region, although values at the contact zone ($H_e \geqslant 0.60$) were slightly higher than in the pure *viridis* or *balearicus* range (Table 1).

Cline analyses

Seven microsatellites with at least one species-specific allele (STRUCTURE) and the mitochondrial marker were selected for cline analyses (Figure 5; Supplementary Table S3). The overall pattern shows two extrinsic effects: that of geographic distances and that of riverbeds (Figure 5). The proportion of *balearicus*-specific alleles decreases smoothly with increasing latitude, with marked drops corresponding approximately to the (historic beds of) Po and Adige Rivers. No *viridis*-specific nuclear alleles were detected south of the Po River. At both transects, the best models had a shared center and a shared slope for six out of the seven nuclear markers (Supplementary Table S4, AICc). The common center was located between the two major rivers (20.0 km northeast of the Po for transect A and 20.5 km for transect B; that is, near the historic Po River bed). Cline widths, calculated as the inverse of the slope, were remarkably narrow (2.3 km for transect A, 2.6 km for transect B; Supplementary Table S3), given that *B. viridis* can migrate 2–10 km in a single year (Blab *et al.*, 1991), but flanked with large introgression tails, expanding northwards of Adige into the *viridis* range (Figure 5). For the mtDNA, the shape of the clines differed strongly from those of nuclear markers, with centers of mtDNA clines located much further north (46.6 km northeast of the Po for transect A; 96.4 km for transect B).

Additional evidence for wide asymmetric introgression and backcrosses

Many cases of cyto-nuclear discordances were detected, with an asymmetric distribution. Of the individuals confidently assigned to *viridis* by nuclear markers (51 inds.), 29% possessed *balearicus* mtDNA (15 inds.), including some from populations distant from the center of the contact (pops. 3–4, 7–8; Figure 1). However, none of those confidently assigned to *balearicus* (23 inds.) presented *viridis* mtDNA (all possessed *balearicus* mtDNA). NEWHYBRIDS also identified many hybrids at localities displaying admixture: populations 22–35 had 42% of F_2 hybrids and 16% of backcrosses, while populations 37–48 had 59% F_2 hybrids and 3–5% of backcrosses (Supplementary

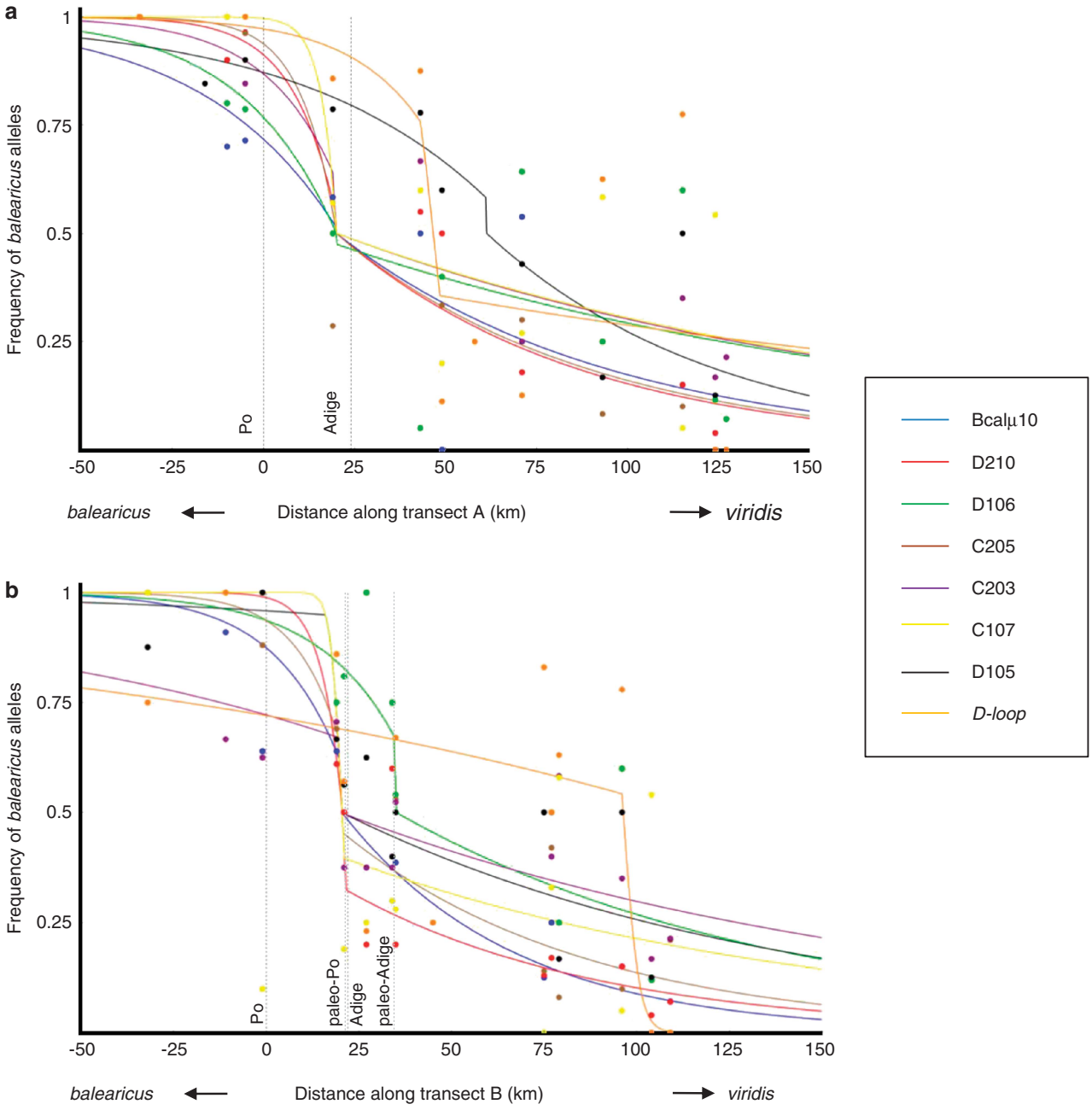

Figure 5 Genetic clines for seven microsatellite loci with species-specific alleles and the mitochondrial control region (*D-loop*) along transects A (**a**) and B (**b**). Relative position of the Po and Adige rivers (current and, if different within the transect, paleo-river beds) is plotted as dashed lines. Transects are the same as in Figure 2, but including only localities where $n \geqslant 4$.

Figure S2). No F_1 hybrids were detected. The geographic distribution of the *Tropomyosin* alleles also provides evidence for introgression, with admixed populations mostly at the center of the contact zone.

DISCUSSION

Differences in the degree of introgression

As our results show, the Italian (*B. balearicus*) and the European green toad (*B. viridis*) come into parapatry in the lower Po and Adige drainages. Despite substantial mitochondrial and nuclear sequence differentiation, accumulated over 1.9 My divergence (Figure 3 and Supplementary Figure S1; see also Stöck *et al.*, 2008a), these two lineages show extensive hybridization in their secondary contact zone. This situation contrasts strikingly with the one documented in Sicily between *B. balearicus* and *B. siculus*, two lineages with a deeper divergence (2.6 My). MtDNA introgression was limited to 40 km in Sicily (Colliard *et al.*, 2010), but stretches up to 130 km in the Po Plain (Figure 2). Similarly, nuclear admixture (that is, >10% assignment probability to the alternative taxon) was almost absent in Sicily, but expands over 40–50 km in the *balearicus/viridis* contact zone (Figure 1).

This contrast is reflected in the patterns of isolation by distance: in Sicily, pairwise F_{ST} reach their highest values (0.50) between the *siculus* and *balearicus* populations immediately adjacent to the contact zone (only ~16 km apart), but increase smoothly with geographic

distance in the *balearicus/viridis* contact zone, reaching their maximum ($F_{ST} = 0.53$) between the most distant populations. Genetic diversity shows a similar contrast: in Sicily, H_e values were the lowest at the *balearicus/siculus* contact zone (Colliard *et al.*, 2010), but were largely uniform over the Po Plain, with slightly higher values ($H_e \geqslant 0.60$) at the *viridis/balearicus* contact zone. This and the advanced degree of hybridization in the Po Plain zone, including many individuals exhibiting a cyto-nuclear discordance, suggest that no major pre- and post-zygotic reproductive barriers limit the gene flow between both species, although detailed bioacoustic studies and examination of Haldane effects (see below) are still missing. Taken together, all population genetics parameters reveal two clearly dissimilar situations: introgression under secondary contacts is still extensive between *B. balearicus* and *B. viridis* (1.9 (2.5–1.3) My divergence) but virtually absent between *B. balearicus* and *B. siculus* (2.6 (3.3–1.9) My divergence).

Although our results are well in line with the relatively few scattered studies in anurans in which the relationship between divergence time and natural degree of hybridization has been studied (see Introduction), the two hybrid zones compared so far do not allow us to draw general conclusions yet. Nevertheless, we note that narrow hybrid zones form in secondary contacts of lineages with Pliocene divergence (>3 My; Szymura, 1993; Hofman *et al.*, 2007, Verardi *et al.*, 2009, Simões *et al.*, 2012), whereas secondary contacts of lineages with more recent Plio-Pleistocene divergence (2.5 My–11 Kya) result in wide(r) hybrid zones with considerable genetic exchange between both gene pools (Santucci *et al.*, 1996, Canestrelli and Nascetti, 2008; Sequeira *et al.*, 2011). While few if any comparative studies within the same radiation have been undertaken in anurans, among urodeles, the *Ensatina* ring species complex presents a famous system, in which 'extant intermediate stages of terminal forms have a nearly continuous range, offering replicated interactions at several stages of divergence' (Pereira and Wake, 2009). Reproductive isolation in *Ensatina* 'is likely to be a byproduct of processes that contribute to overall [nuclear] genetic divergence, such as time in geographic isolation' (Pereira *et al.*, 2011), and recent evidence supports 'asymmetric reproductive isolation between terminal forms' of the ring (Devitt *et al.*, 2011).

Beyond divergence time, additional specificities of the two green toad hybrid zones may contribute to the observed introgression differences. Island populations, like that of *B. siculus*, can experience increased drift and selection and thus accelerated rates of molecular evolution (for example, Woolfit and Bromham, 2005), which could enhance the incompatibility between *B. balearicus* and *B. siculus* gene pools. Moreover, we assume that the time since toad lineages came first into secondary contact in Sicily dates at least back to low sea levels during the Last Glacial Maximum (20 Kya; allowing *B. balearicus* to colonize Sicily). Signatures of population expansion (43–3 Kya; see above; and river translocations, see below) point to similar, and thus an overlapping time period for the first contact between *B. balearicus* and *B. viridis* in the Po Plain. Therefore, the age of contact zones would hardly explain the contrasting introgression patterns.

Hybrid zone movement, geography and colonization history

Gene flow in the Po Plain seems barely restricted by genomic incompatibilities and rather reflects the history of colonization and dispersal effects as well as fine scale influences of geographic features.

Interestingly, the clines of nuclear markers are asymmetric, with a long tail on the *viridis* side (Figure 3). A tail of clines of unlinked neutral markers with apparent unidirectional introgression across the zone (Moran, 1981, cited in Buggs, 2007) is typical of a 'moving hybrid zone'. Barton and Hewitt (1985) suggested that evidence for hybrid zone movement should be based on many neutral alleles, introgressing in the same direction. This is the case for *balearicus* microsatellite alleles and suggests zone movement as partial explanation for the introgression asymmetry and tail at the *viridis* side of the zone in the Po Plain. This evidence is further supported by an asymmetry of nuDNA vs mtDNA introgression, again with a tail at the *viridis* side of the hybrid zone. Contrasting with most nuclear markers that share a common center (20 km north of the modern Po river), the mtDNA cline centers much further north (Figure 5), in line with the patterns of cyto-nuclear discordances: we found many *balearicus* mtDNAs in a *viridis* nuclear background but not the reverse.

Beyond introgression asymmetry and isolation by distance, the *balearicus/viridis* hybrid zone reflects signatures of physical barriers (Figures 2 and 5) to gene flow imposed by the current and historic position of large rivers (Po and Adige). Partial Mantel-tests could assign 36% of the variance in pairwise F_{ST} to the number of main (or historic) rivers (0, 1 or 2), separating populations. Cline analyses also showed marked drops in the frequency of *balearicus* specific alleles, corresponding to these main, current or historic, rivers (Figure 5). Intriguingly, all hybrid populations showing nuclear introgression (Figure 1) are almost perfectly delimitated to the south by the modern Po river, and to the northeast by the ancient Po and Adige riverbeds, which, some 5 Kya, were situated 20–40 km farther northeast than today (pink in Figures 1 and 2; Piovan *et al.*, 2010). As the Mantel tests supported, the locations of historical riverbeds were only slightly less important than modern ones in accounting for present day population structure (Table 3).

All of this suggests the following scenario for this secondary contact zone. Since the Last Glacial Maximum (LGM), the Po Plain vegetation changed from a natural steppe-like to a densely forested habitat. This was followed some 4–5 Ky ago by human deforestation (Amorosi, 2004), which presumably facilitated green toad range expansion. The region was progressively colonized by *balearicus* and *viridis* from their glacial refugia, respectively, south and east of the study area. The contact zone might have been established at the Po and/or Adige paleo-riverbeds, which at this time posed faster running river barriers than today (Fontana *et al.*, 2008), with relatively few, but some migrants overcoming them (for example, *balearicus* may have expanded further north). A 20–40 km southward river translocation into the modern beds then trapped some *balearicus* populations northeast of the present-day Po River. Continuous gene flow from incoming northern *viridis* colonizers progressively diluted local *balearicus* genomes, now isolated by the Po River from the main range of *balearicus*. The long tail of mtDNA and nuDNA clines in the north might be a result of this influx of *viridis* genes into the hybrid zone, explicable as '*balearicus* dilution' itself and/or by truly south-westwards migrating *viridis* populations. In any case, this dynamic, apparently ongoing process exhibits the clear features of a 'moving hybrid zone'. In accordance with a scenario of '*balearicus* dilution', NewHybrids analyses did not detect any pure *balearicus* or F_1 genotypes north of the Po River, suggesting instead an advanced state of nuclear admixture ('F_2' and backcrosses; Supplementary Figure S2). The absence of 'F_1' suggests that the individuals labeled 'F_2' might in fact also represent backcrosses. Most importantly, these data imply that at least some hybrids become adult and fertile, as they successfully reproduce. This scenario would suggest a minimal age of 5 Ky for the hybrid zone (that is, >2500 generations, assuming a 2-year generation time; Stöck *et al.*, 2008b), as inferred from the last

major Po River translocation. The further reaching mitochondrial than nuclear introgression of *balearicus* into a *viridis* background might further indicate a smaller female effective size in *viridis* invaders, possibly stemming from sex differences in dispersal ability. A male-biased dispersal is expected to increase male effective population size at the front of invading populations, favoring mtDNA over nuclear introgression (Petit and Excoffier, 2009). We do not know whether slight size differences between the green toad lineages involved (Stöck *et al.*, 2008b) could skew the mating preferences as, for example, observed within *B. bufo* for differently sized males (Davies and Halliday, 1979). Another alternative that could explain nuDNA/mtDNA introgression asymmetry might stem from sex differences in hybrid fitness or fecundity. Haldane's rule predicts lower hybrid fitness in the heterogametic sex. Several taxa of the *B. viridis* group, probably including *B. balearicus*, have a male heterogametic (XY) sex determination system (Stöck *et al.*, 2013). Although Haldane's rule might be less prevalent in taxa with homomorphic sex chromosomes, too few non-model hybrid organisms have been studied, especially in natural systems of amphibians, to exclude this scenario (Schilthuizen *et al.*, 2011).

CONCLUSIONS

Our data show a striking contrast in the degree of hybridization between closely related green toad lineages in secondary contact. In comparison with the system of greater divergence (Sicily, 2.6 My), the North-Italian green toad hybrid zone (of lineages diverged 1.9 My) exhibits a much wider and asymmetric introgression at nuclear and mitochondrial levels. Gene flow in this apparently dynamic system seems mainly constrained by local geographic barriers (large rivers), and less by intrinsic genomic incompatibilities. All of this suggests that reproductive isolation during incipient speciation increases gradually with the time of divergence (up to a certain threshold; for example, Nosil and Feder, 2012), and might be driven by complex genomic processes rather than single speciation genes. Our study represents a contribution toward comparative studies of secondary contacts of closely related anuran lineages. It is well in line with scattered research in other anuran species, with examples among discoglossoids, bufonids, hylids and ranids, but has the advantage to compare lineages with different divergence times from the same radiation.

DATA ARCHIVING

Genotype data available from the Dryad Digital Repository: doi:10.5061/dryad.85pr1. GenBank accessions for the *D-loop* and *alpha-Tropomyosin* have in part been published by Stöck *et al.* (2008a) and Colliard *et al.* (2010); new data in the present paper have accessions: KJ532478-KJ532515 (alpha-Tropomyosin) and KJ532516-KJ532802 (D-loop).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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