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New insights on phylogeography and distribution of painted frogs (*Discoglossus*) in northern Africa and the Iberian Peninsula

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Abstract. Painted frogs (*Discoglossus*) contain five to six species of Western Palearctic anurans that are mainly distributed in allopatry. We here provide the first comprehensive assessment of the phylogeography of the Moroccan species *D. scovazzi* and geographically characterize its contact zone with *D. pictus* in Eastern Morocco. *Discoglossus scovazzi* shows, in general, a weak phylogeographic structure across Morocco on the basis of mitochondrial DNA sequences of the cytochrome *b* gene, with only populations centered in the Atlas Mountains characterized by the presence of slightly divergent haplotypes. In eastern Morocco, all populations east of the Moulouya River were clearly assignable to *D. pictus*. This species was also found along the Mediterranean coast west of the Moulouya, in the cities of Nador and Melilla, suggesting that not the river itself but the wide arid valley extending along much of the river (except close to the estuary) acts as a possible distributional barrier to these frogs. No sympatry of *D. scovazzi* with *D. pictus* was observed, and all specimens were concordantly assigned to either species by DNA sequences of cytochrome *b* and of the nuclear marker RAG1. Species distribution models of the two taxa show largely overlapping areas of suitable habitat, and the two species' niches are significantly more similar than would be expected given the underlying environmental differences between the regions in which they occur. Comparative data are also presented from the southern Iberian contact zone of *D. galganoi galganoi* and *D. g. jeanneae*. These taxa showed less clear-cut distributional borders, extensively shared RAG1 haplotypes, and had instances of sympatric occurrence on the basis of cytochrome *b* haplotypes, in agreement with the hypothesis of a yet incomplete speciation. In this wide contact zone area we found mitochondrial sequences containing double peaks in electropherograms, suggesting nuclear pseudogenes or (less likely) heteroplasmy, possibly related to the ongoing admixture among the lineages.

Keywords: Alytidae, Amphibia, biogeography, heteroplasmy, Morocco, niche overlap, NUMTs, species distribution models.

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Introduction

Painted frogs (*Discoglossus* Otth, 1837) are representatives of an ancient anuran clade, the Alytidae Fitzinger, 1843, with an exclusively Western Palearctic distribution. Among extant frogs, the sister taxon of *Discoglossus* is *Latonia nigriventer* (Mendelssohn and Steinitz, 1943) from Israel (Biton et al., 2013), and the *Discoglossus-Latonia* clade is sister to the midwife toads, *Alytes* Wagler, 1830 (Roelants and Bossuyt, 2005; Roelants et al., 2007; Biton et al., 2013). *Discoglossus* currently contains 5-6 extant species: *D. montalentii* Lanza, Nascetti, Capula and Bullini, 1984 from Corsica, *D. sardus* Tschudi, 1837 from Corsica, Sardinia, and some smaller Mediterranean islands; *D. pictus* Otth, 1837 from Sicily, Malta, Tunisia, Algeria, and eastern Morocco, introduced to southern France and eastern Spain (Catalonia Region); *D. scovazzi* Camerano, 1878 from Morocco; and *D. galganoi* Capula, Nascetti, Lanza, Bullini and Crespo, 1985 and *D. jeanneae* Busack, 1986 from Iberia. The taxon *jeanneae* is often considered a subspecies of *D. galganoi* (Zangari, Cimmaruta and Nascetti, 2006; Speybroeck, Beukema and Crochet, 2010; Pabijan et al., 2012; Vences and Grossenbacher, 2012). We herein follow this view which however might be challenged by future detailed analysis of gene flow among these taxa across their contact zone.

The phylogeny and phylogeography of *Discoglossus* has been subject of numerous studies (Lanza et al., 1984, 1986; García-Paris and Jockusch, 1999; Martínez-Solano, 2004; Real et al., 2005; Zangari, Cimmaruta and Nascetti, 2006; Velo-Antón et al., 2008; Pabijan et al., 2012; Biton et al., 2013). The available evidence strongly suggests *D. montalentii* being the sister taxon of all other species of *Discoglossus*, and a sister group relationship of *D. g. galganoi* and *D. g. jeanneae*. Most probably, *D. pictus* and *D. sardus* are sister to each other, and *D. scovazzi* is sister to the *galganoi-jeanneae* clade (Pabijan et al., 2012).

Besides the broadly sympatric *D. montalentii* and *D. sardus* in Corsica, the distribution areas of distinct *Discoglossus* taxa abut in two regions: on one hand, the ranges of *D. g. galganoi* and *D. g. jeanneae* contact each other across central Spain (García-Paris and Jockusch, 1999; Martínez-Solano, 2004; Real et al., 2005; Velo-Antón et al., 2008), and on the other hand, those of *D. pictus* and *D. scovazzi* abut in eastern Morocco, roughly in the area of the Moulouya River (Zangari, Cimmaruta and Nascetti, 2006). The geographical distribution of the two taxa in this region is at present poorly understood, because *D. scovazzi* has long been considered to be a subspecies of *D. pictus* (e.g., Lanza et al., 1986). Therefore natural history and distributional information of North African *Discoglossus* populations have usually been recorded under a single species name, *D. pictus* (e.g., Salvador, 1996), and the species identity of most records in eastern Morocco is so far uncertain (Beukema et al., 2013; Reques et al., 2013). Although a number of karyological (Odierna et al., 1999; Amor et al., 2007) and molecular data (Lanza et al., 1986; Zangari, Cimmaruta and Nascetti, 2006) have become available from North African *Discoglossus*, their phylogeography is so far understudied, especially compared with the Iberian taxa.

Here, we present the first phylogeographic analysis of *D. scovazzi* across its entire range, focusing on eastern Morocco where its range contacts that of *D. pictus*, and provide new data on the distribution and phylogeography of *D. g. galganoi* and *D. g. jeanneae* in southern Iberia. Our results are based on a large number of newly determined sequences of one mitochondrial and one nuclear gene from these four taxa, and on modelling environmental niches of *D. pictus* and *D. scovazzi* based on a newly compiled locality database for these species.

Materials and methods

Field work and sampling

Sampling was carried out from 2010-2013 in southern Spain and in Morocco, targeting specifically the putative contact zones of *galganoi-jeanneae* and *pictus-scovazzi* following

data of Zangari, Cimmaruta and Nascetti (2006). Additional tissue samples or extracted DNA were available from different collections, or were collected oportunistically by collaborators in the framework of other projects. Tissue samples included femur muscle of roadkills or preserved voucher specimens (in particular from Morocco), toe clips, and tadpole fin clips. See online Supplementary Material: Table S1 for a complete list of localities and geographical coordinates.

Molecular methods

Samples were extracted using a standard salt extraction protocol (Bruford et al., 1992). Fragments of one mitochondrial and one nuclear gene were amplified using newly developed specific primers: a fragment of the mitochondrial cytochrome *b* (COB) gene was amplified with the primers CytbA-Disco (CCCTGAGGACAGATATCRTTTTGAGG) and CytbC-Disco (CTACTGGTTGRCCCCCGATCCAGG T) with a PCR protocol consisting of an initial step of 90 seconds at 94°C, followed by 35 steps of 94°C (30 s), 53°C (45 s), 72°C (90 s) and a final elongation step of 10 min at 72°C. A fragment of the nuclear recombination-activating gene I (RAG1) was amplified using Disco-Rag1-F1 (ATCCAGTGGAAGCAATTTCG) and Disco-Rag1-R1 (CTCAGTGTGGCACCTGGTTA) with 120 seconds at 94°C, followed by 40 steps of 94°C (20 s), 58°C (50 s), 72°C (180 s) and a final elongation step of 10 min at 72°C. For a subset of individuals, an additional segment of COB (extending in the 5′ direction and partly overlapping with the first fragment) was amplified using Cytb-DiscoF (ATTGTTAATAACTCATTTATTG) and Cytb-DiscoR (ACTTTCTCTAAGTTTGAGT). In these cases sequences submitted to Genbank were contigs of the two segments. Because the second segment was only available for few specimens it was not included in the further analyses. From a few samples and to verify the presence of double peaks in electropherograms of mtDNA sequences (see below) we also amplified a segment of the mitochondrial Cytochrome Oxidase Subunit I (COX1) gene using primers COI-VertF1 and COI-VertF2; primer sequences in Vences et al. (2012).

PCR products were treated with Exonuclease I (New England Biolabs) and Shrimp Alkaline Phosphatase (Promega) to inactivate remaining primers and dNTPs, and then were cycle-sequenced using dye-labeled terminators (Applied Biosystems) with the amplification primers. All RAG1 amplicons were sequenced in both directions while the majority of COB amplicons were sequenced with the forward primer only. A selection of COB amplicons characterized by double peaks (see below) were ligated into the pCR Blunt vector included in the Zero Blunt PCR Cloning Kit (Invitrogen) and transformed into competent *E. coli* cells, following manufacturer's protols. After plasmid isolation via alkaline lysis, plasmids were sequenced using M13 primers. For this analysis we chose three samples with particularly clear double-peak signal in the COB sequences obtained through direct sequencing of PCR products.

Sequences were resolved on an ABI 3130XL automated DNA sequencer (Applied Biosystems). Chromatographs were checked and sequences were edited and assembled using Codon-Code Aligner (v2.0.6, Codon Code Corporation). All newly determined sequences were submitted to GenBank (accession numbers KF644587-KF645288).

DNA sequence analysis

Sequences were aligned using the Clustal algorithm in MEGA, vs. 5 (Tamura et al., 2011). RAG1 haplotypes of nuclear gene sequences were inferred using the PHASE algorithm (Stephens, Smith and Donnelly, 2001), implemented in DnaSP v5 (Librado and Rozas, 2009), and the same program was used to calculate values of genetic diversity per population. In some cases, haplotype reconstruction was not unambiguously possible; we nevertheless decided to include these haplotypes (the pair with the highest score for each individual) in the network analysis because a wrongly inferred haplotype would only slightly alter the number of haplotypes and their placement in the haplotype network, and thus would probably not influence our analyses (which relies mainly on identifying major haplogroups and their geographical distribution) in a relevant way.

During our analysis of COB sequences, we noted distinct double peaks in the electropherograms, i.e., overlapping peaks of about half the height of normal peaks. In some cases these were less distinct so that the total number of "heterozygous" individuals could not be determined with full certainty, although it certainly exceeded 50 individuals and mainly affected those from the South of the Iberian Peninsula. This phenomenon suggested occurrence of nuclear copies of mitochondrial DNA (NUMTs) and/or heteroplasmy. Clarifying this issue with full reliability was outside the scope of the present study and would require intensive additional molecular work. We here only performed a few experiments to obtain preliminary information on the possible causes of the observed double peaks. For three of the "heterozygous" specimens with particularly obvious double peaks, we sequenced the different sequences from the heterogeneous amplicons via cloning (see above), but for the others, in order to include these sequences in the haplotype network reconstruction, we used the PHASE algorithm to separate them. We are aware that this procedure strictly speaking is invalid, as more than two variants might be present per sample. This restriction in particular applies to 61 *D. galganoi* sequences for which we scored more than 2 heterozygote positions. Yet, we consider this approach to be more accurate than simply using the raw sequences for reconstruction of a phylogenetic tree or haplotype network, by either using IUPAC ambiguity codes (tree only) or excluding the heterozygous sites (tree or network).

Previous studies (e.g., Fromhage, Vences and Veith, 2004; Zangari, Cimmaruta and Nascetti, 2006) have shown that phylogenetic analyses based on one or two short gene fragments are of insufficient resolving power to reliably infer interspecific relationships among *Discoglossus* species. Concatenated and species tree analyses of multigene DNA sequence data sets have provided concordant reconstructions of the evolutionary history of this genus (Pabijan et al., 2012; Biton et al., 2013), and we therefore refrained from phylogenetic analyses of our COB and RAG1 data set and

instead opted for visualizing patterns of differentiation in the form of haplotype networks.

Haplotype network reconstructions for COB and RAG1 were performed under statistical parsimony (Templeton, Crandall and Sing, 1992) using the software TCS v1.21 (Clement, Posada and Crandall, 2000). To analyse the relationship between genetic (mean number of pairwise nucleotide differences) and geographical distance (km) between populations (often referred to as isolation-by-distance), we performed a Mantel's tests (Mantel, 1967) using ARLEQUIN v3.11 (Excoffier, Laval and Schneider, 2005). The geographical distances between localities were calculated from GPS coordinates in the software GENALEX v6 (Peakall and Smouse, 2006). Data were permuted 1000 times to estimate the 95% upper tail probability of the matrix correlation coefficients.

Species distribution modelling

A total of 456 distribution records of North African *Discoglossus* were assembled from literature, museum collections and own fieldwork (see online Supplementary Table S2). The distribution records went through a process of filtering that removed duplicate records within unique grid cells in ENMtools 1.3 (Warren, Glor and Turelli, 2010) and also reduced sampling bias by using a kernel density grid as implemented in the Java program OccurrenceThinner v1.0.4 (Verbruggen et al., 2013). After filtering, the final dataset used for modelling consisted of 218 distribution records (*D. scovazzi*: $n = 119$, *D. pictus*: $n = 99$; see online Supplementary Tables S3-S4).

All available bioclimatic variables were downloaded from the WorldClim database version 1.4 (http://www.worldclim.org; Hijmans et al., 2005) at a resolution of 2.5 arc minutes (nearly 5×5 km). Past climate data for the Last Glacial Maximum (LGM; ca. ~21 000 years BP) was obtained from the WorldClim database at the same resolution. Two general atmospheric circulation models (GCM) were used to generate the LGM scenarios: the Community Climate System Model (CCSM) and the Model for Interdisciplinary Research on Climate (MIROC). The two GCMs were averaged using ArcGIS 10 (ESRI). Collinearity of the initial variables was measured by Pearson's correlation coefficient in ENMtools v1.3 (Warren, Glor and Turelli, 2010). A total of seven variables, all of which had a correlation degree lower than 0.75 (Pearson coefficient) were retained. The final set of environmental predictor variables used for the species distribution models (SDM) consisted of: Annual Temperature (BIO1), Temperature Seasonality (BIO4), Maximum Temperature of Warmest Month (BIO5), Mean Temperature of Driest Quarter (BIO9), Annual Precipitation (BIO12), Precipitation Seasonality (BIO15) and Precipitation of Warmest Quarter (BIO18).

The SDMs were generated by the presence/background algorithm implemented in Maxent, version 3.3.3k (Phillips, Anderson and Shapire, 2006). Maxent was used with default settings (convergence threshold = 0.00001, maximum number of iterations = 500 and $\beta j = 1$), partitioning the geographical records between training and test samples (default settings). We defined a background for each species by drawing a 200 km buffer around all distribution records and subsequently projected the models onto a larger area.

The average of ten pseudo-replicated models with randomly selected test samples was used to produce SDMs, which were plotted in logistic format. The final models were reclassified in ArcGIS 10 (ESRI) into binary presence-absence maps based on the assumption that ten percent of the records were either wrongly identified or georeferenced, meaning, that the 10% of model outputs with the lowest predicted probabilities fall into the 'absence' region of the threshold model, and 'presence' regions include the 90% of distribution records with the highest model values (Raes et al., 2009). All models were tested with receiver operating characteristics (ROC) curve plots, and the area under the curve (AUC) of the ROC plot of ten models was taken as a measure of the overall fit of each model.

Additionally, we used null-models to test for significance of the SDMs. We generated 100 null distributions of random points in the study area using ENMtools (Warren, Glor and Turelli, 2010) for each of the two species, with the number of random points equal to the actual number of distribution records used for SDM. The null-models were created and assessed following Raes and ter Steege (2007).

Quantifying niche overlap

In order to quantify the degree of ecological differentiation between *D. pictus* and *D. scovazzi*, we employed a multivariate analysis framework proposed by Broennimann et al. (2012) implemented in R (R Development Core Team, 2008), using the same climate variables, distribution records and background as for SDM. Following this framework we computed multivariate environmental niche overlaps between *D. pictus* and *D. scovazzi* employing the two best performing ordination techniques (Broennimann et al., 2012): (1) Principal Component Analysis (PCA) calibrated on the entire environmental space of the study area (termed PCA-env; Broennimann et al., 2012), and (2) Ecological Niche Factor Analysis (ENFA) (Hirzel et al., 2002). The framework by Broennimann et al. (2012) implements a modified niche similarity and niche equivalency tests *sensu* Warren, Glor and Turelli (2008) and calculates niche overlap for pairs of species using Schoener's *D* (Schoener, 1970).

Results

Genetic analyses

We determined new COB sequences from 395 samples of the four target taxa and RAG1 sequences from 374 samples. All of these 769 sequences (not counting those obtained by cloning) were used to identify lineages and determine distribution ranges, but for further analysis only subsets of sequences were used. Numerous sequences were exceedingly short or

had longer sections with missing data and were therefore excluded from the set submitted to Genbank (a total of 702 sequences submitted: 359 COB and 343 RAG1). For final analyses, all sequences were cut to remove sections with missing data at the end or beginning. Because for haplotype phasing, alignments must not contain such missing data, we additionally excluded a few sequences so that the final alignments used for analysis contained 357 sequences of 430 bp for COB, and 342 sequences of 361 bp for RAG1. Geographical distribution of samples and their assignment to species according to the molecular data is shown in fig. 1. The haplotype networks for the different taxa are shown in figs 2-4, and numbers of sequences per locality are listed in Supplementary Table S1.

Both COB and RAG1 congruently separated haplotypes of *D. scovazzi* (fig. 2) and *D. pictus* (fig. 3) into two networks. In no case did a population share haplotypes belonging to the two species, no individual had a COB haplotype of one and RAG1 haplotypes of the other species, and no heterozygotes for RAG1 haplotypes of the two species were found. *Discoglossus g. galganoi* and *D. g. jeanneae* were separated by COB into two unconnected networks, whereas the RAG1 sequences of both taxa were

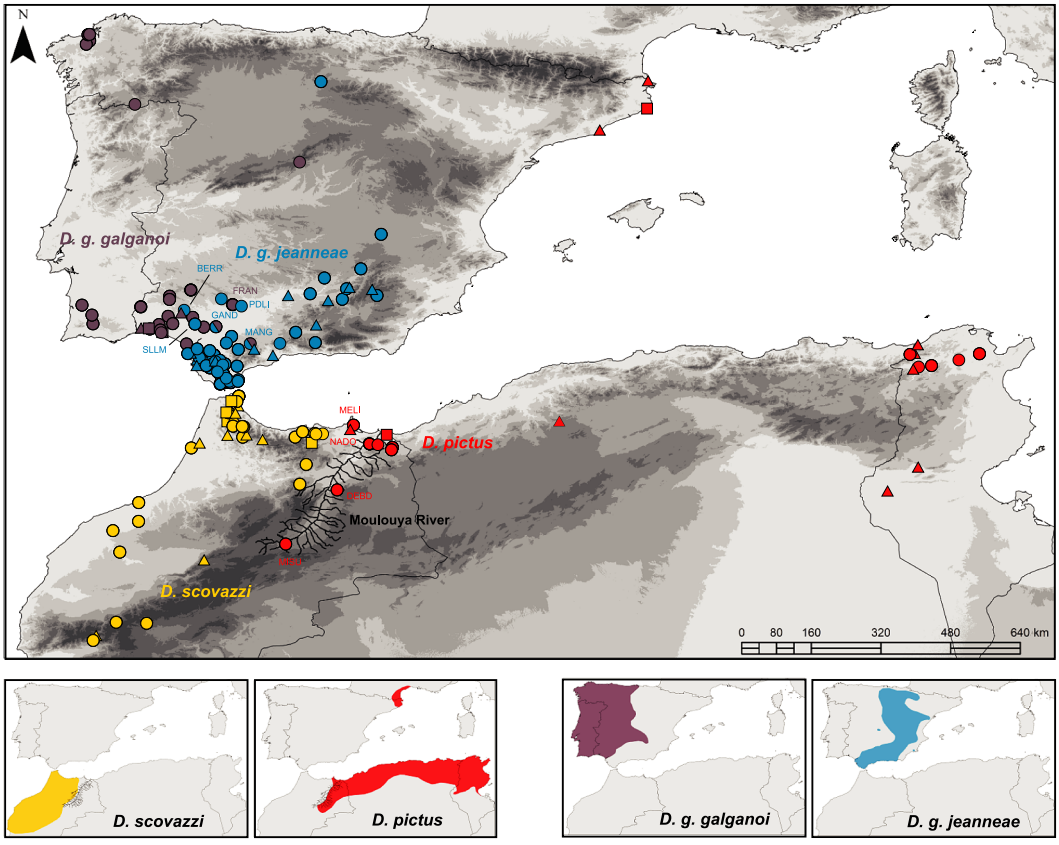

Figure 1. Distribution of sampling sites of *Discoglossus* taxa included in this study, with taxon assignment based on COB and RAG1 sequences. Circles: COB and RAG1 sequences available; triangles, only RAG1; squares, only COB. *D. g. galganoi* and *D. g. jeanneae* could not be distinguished based on RAG1 alone. Therefore, the taxon assignment of individuals from Iberia, for which only RAG1 sequences were available, was based only on geography. Two bicolored circles in Iberia indicate locations were mtDNA haplotypes of both taxa was observed in syntopy. Localities discussed in the text are marked with their four-letter codes (as explained in the text and/or in Table S1). The lower row of inset maps shows the approximate distribution of the four taxa according to the IUCN Red List (www.redlist.org, accessed April 2014); note that the distribution of *D. pictus* extends further into Malta, Gozo, and Sicily (not shown in inset map). This figure is published in colour in the online version.

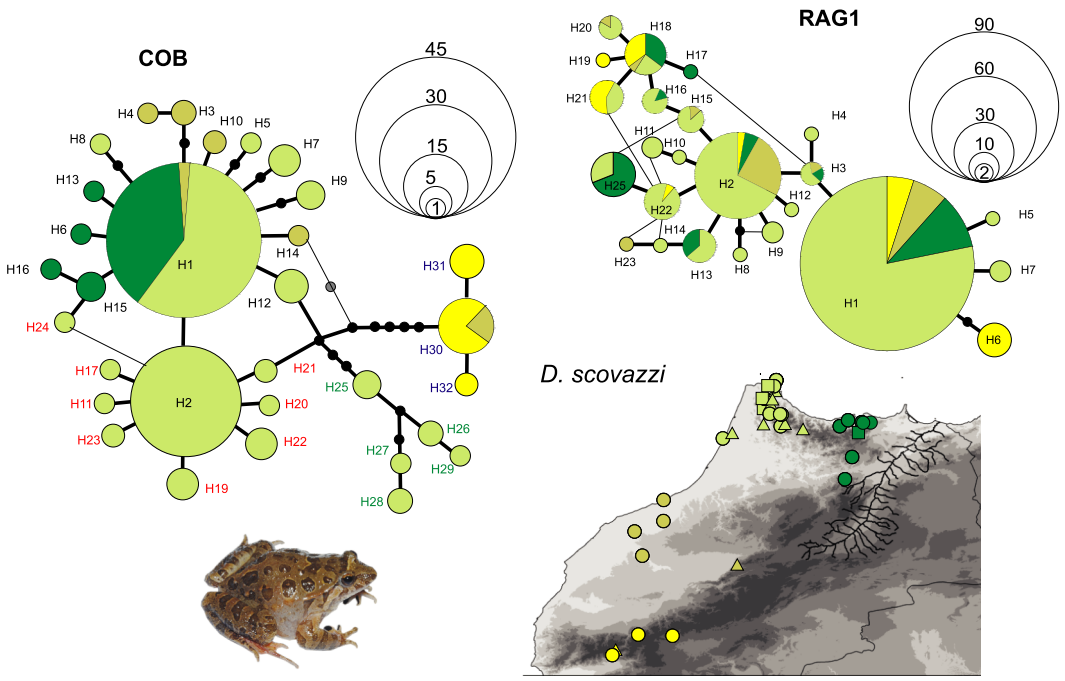

Figure 2. Haplotype networks for *Discoglossus scovazzi*, based on COB and RAG1 sequences. Colors represent ad-hoc definitions of major geographic areas, to visualize the distribution of the haplotypes. This figure is published in colour in the online version.

arranged in a single network with only five haplotypes, three of which were shared (fig. 4).

The isolation-by-distance analysis, excluding *D. pictus* for which our sampling is too patchy for reliable interpretation, revealed a significant correlation for *D. scovazzi*, and *D. g. galganoi*, but not for *D. g. jeanneae*, in COB, and for none of these lineages in RAG1 (details in Supplementary Material).

A closer look at the *D. scovazzi* COB network (fig. 2) suggests the presence of a somewhat distinct haplotype cluster in the High Atlas populations (colored in yellow in the network), differing by a minimum of seven mutational steps from other haplotypes. Of the three haplotypes we determined in the High Atlas, the most common one (H30), however, was also found in the lowlands between Marrakech and Casablanca. *Discoglossus pictus* showed a clear structure between populations from Tunisia, the population from Catalonia (Spain) (PALA), and the Moroccan populations (fig. 3), and this is in agreement with previous data indicating at least three distinct mitochondrial lineages within this species (Zangari, Cimmaruta and Nascetti, 2006).

Our intensive sampling in eastern Morocco roughly confirmed the basin of the Moulouya River constituting the barrier between *D. pictus* (east) and *D. scovazzi* (west). In the coastal areas, all populations sampled east of the Moulouya had *D. pictus* haplotypes only, and we also only found this species in the isolated Debdou Massif (locality DEBD) and in the upper Moulouya at Saïda, south of Missour (locality MISU). At the coast, however, we found haplotypes of *D. pictus* also in Melilla (locality MELI; several COB and RAG1 sequences) and probably in Nador (NADO; only a short poor-quality RAG1 sequence available, not included in Table S1), both located west of the Moulouya River (see fig. 1). About 30 km west from these localities, in the hills and mountains between Midar and Al Hoceima, all populations sampled were identified as *D. scovazzi*, while no *Discoglossus* were found in between Nador and Midar.

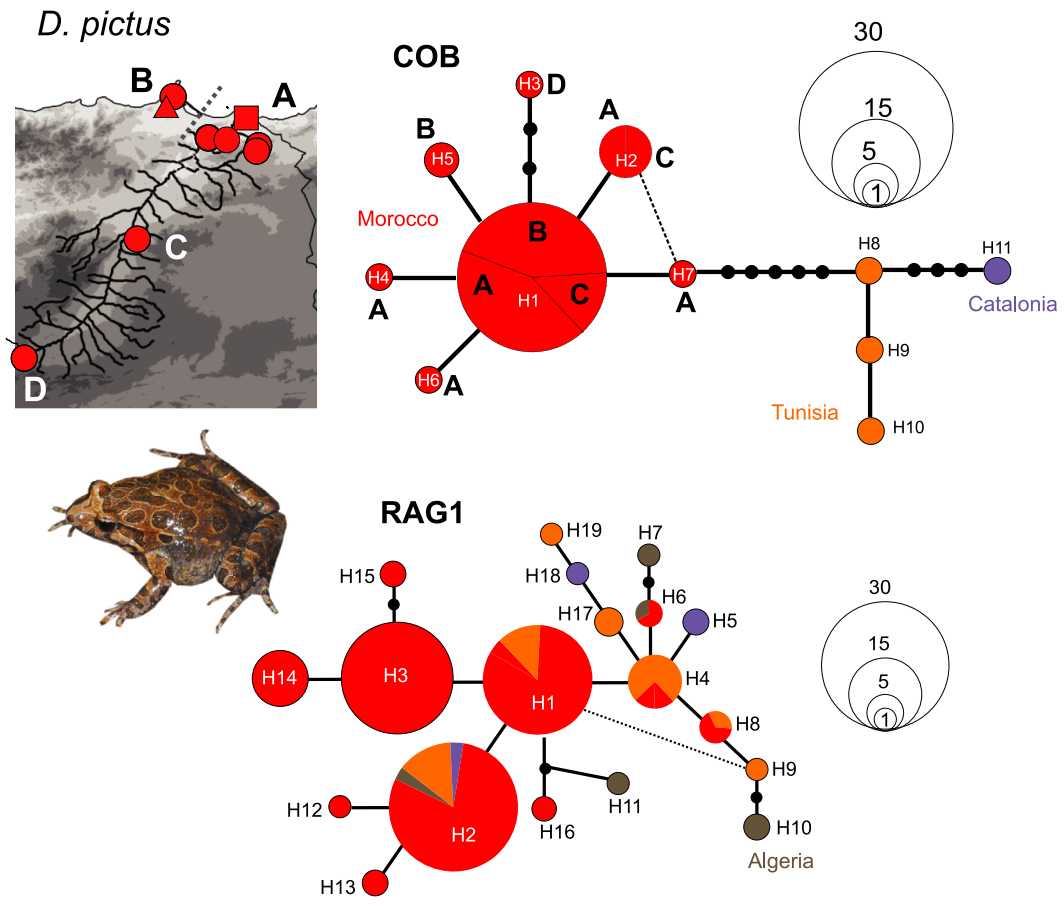

Figure 3. Haplotype network for *Discoglossus pictus* based on COB and RAG1 sequences. Colors represent ad-hoc definitions of major geographic areas, to visualize the distribution of haplotypes; i.e., in Morocco (red) Tunisia (orange) and Catalonia region, Spain (purple), of which only the Moroccan sites are shown on the map. Letters A-D on the map correspond to the letters in the COB network above, indicating that populations east (A, C, D) and west (B) of the Moulouya River share the same main COB haplotypes H1. This figure is published in colour in the online version.

In southern Spain, we found COB haplotypes of both *D. g. galganoi* and *D. g. jeanneae* in two populations: at Gandul near Alcala de Guadaira (locality GAND), close to an area where syntopy of the two lineages had been observed before (Zangari, Cimmaruta and Nascetti, 2006), and about 26 km north of Ronda (locality MANG). Furthermore, in several areas, single samples assigned to either of the two lineages occurred in close proximity and apparently without clear-cut range borders between them, for instance in the area between Constantina and Palma del Rio (locality PDLI assigned to *jeanneae*, FRAN to *galganoi*) or at Berrocal (BERR) and Sanlucar la Mayor (SLLM), where *jeanneae* haplotypes occurred largely surrounded by localities with *galganoi* haplotypes (see Supplementary Table S1 for locality codes and geographical coordinates; not indicated in map due to the very dense sampling in this area).

To obtain a first understanding of the pattern and underlying causes for the observed double peaks in the mitochondrial COB sequences, we first amplified an additional mitochondrial gene (COX1) from a series of specimens and found that double peaks were present also in those sequences. Furthermore, we carefully re-extracted DNA from a series of freshly collected samples to exclude sample contamination. Re-

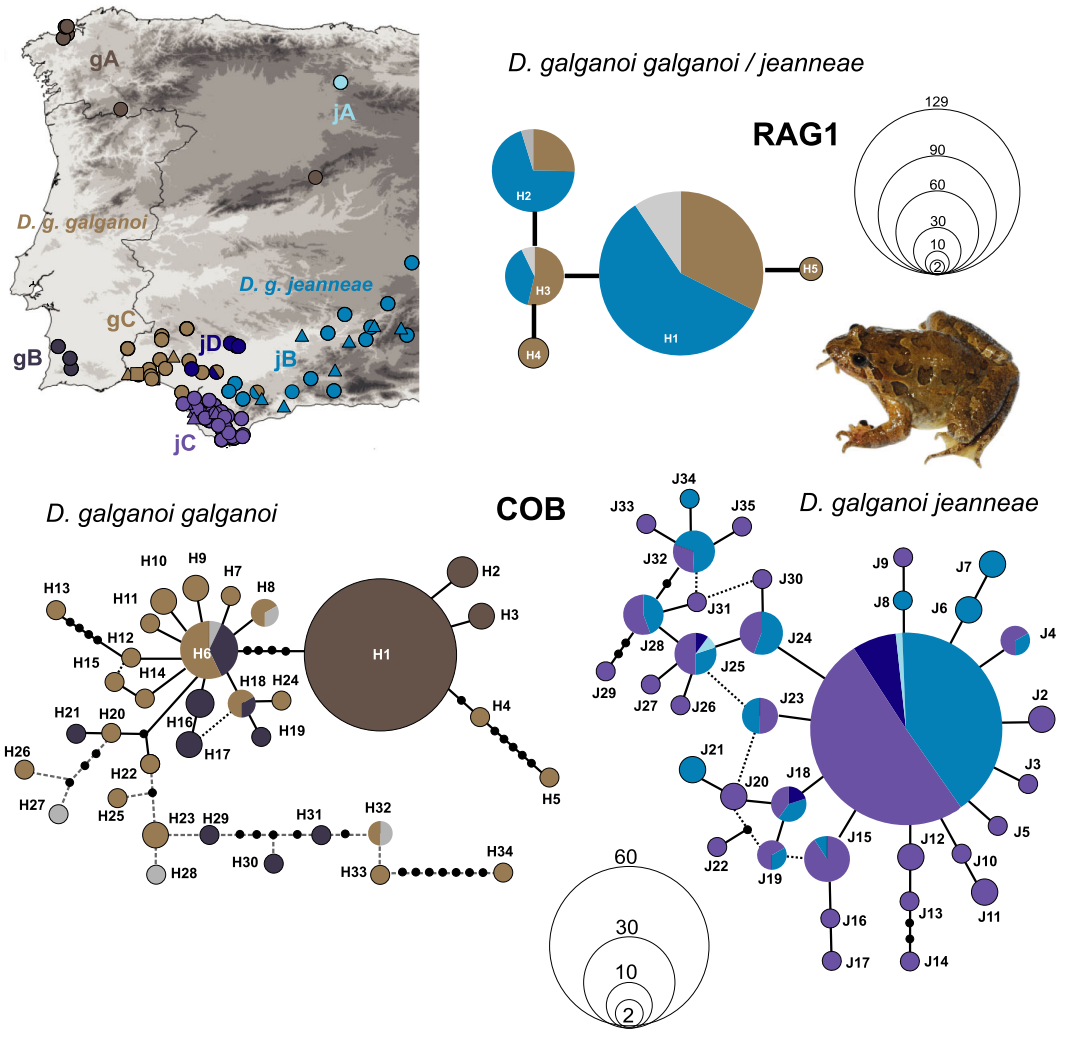

Figure 4. Haplotype network for *Discoglossus g. galganoi* and *D. g. jeanneae*, based on RAG1 (above) and COB sequences (below). Letters gA-gC and jA-jD denote populations from ad hoc defined geographical regions which are color coded in the map and in the COB networks, to indicate the geographical distribution of the haplotypes. In the RAG1 network, colors (brown vs. blue) denote the proportion of *D. g. galganoi* vs. *D. g. jeanneae* individuals sharing a particular haplotype; grey denotes individuals for which no assignment to either of the main mtDNA lineages is possible due to lack of COB sequences. Note that sampling of these taxa is concentrated on the southern parts of the species' ranges. This figure is published in colour in the online version.

sequencing from these new extractions yielded the identical sequences with double peaks. After using the Phase algorithm to separate the obtained "haplotypes", these always clustered with the same lineage (either both with *galganoi*, or both with *jeanneae*). When we examined a large number of samples of *D. g. galganoi* from localities in Galicia (north-western Spain), far from the contact zone with *D. g. jeanneae*, we could not find double peaks in any of the COB sequences from this region.

We then re-amplified COB from three specimens from southern localities characterized by a particular intensity of such double peaks. The PCR products were firstly sequenced directly and secondly also cloned into plasmids. Four to five isolated plasmids per sample were sequenced. In two cases (MV2894, a speci-

men without reliable locality information included because of its particularly obvious double peaks, but not further considered for the phylogeographic analysis, and DG_GAND02 from Gandul) we found clones clustering with both *jeanneae* and *galganoi*. For DG_GAND02, three of the four clones clustered with *galganoi* and one with *jeanneae*. This individual initially had been placed in *galganoi*, when the taxon assignment was based on the sequence obtained by direct sequencing of the PCR product and this sequence contained many double peaks. For the third individual, DG_SDMO01, all four sequences derived from clones, as well as the regularly obtained one, clustered with *jeanneae*. All COB sequences obtained from clones translated into amino acids without gaps or stop codons.

Species distribution modelling and niche overlap

Maxent produced SDMs of moderate predictive accuracy (following Swets, 1988), according to the average test AUC for the present and past models (*D. pictus*: average AUC = 0.770 ± 0.057; *D. scovazzi*: average AUC = 0.801 ± 0.050). All SDMs (fig. 5) performed statistically significantly better than random. The main predictor variables differed between *D. pictus* (BIO4 = 54.1%, BIO12 = 14.9%) and *D. sco-*

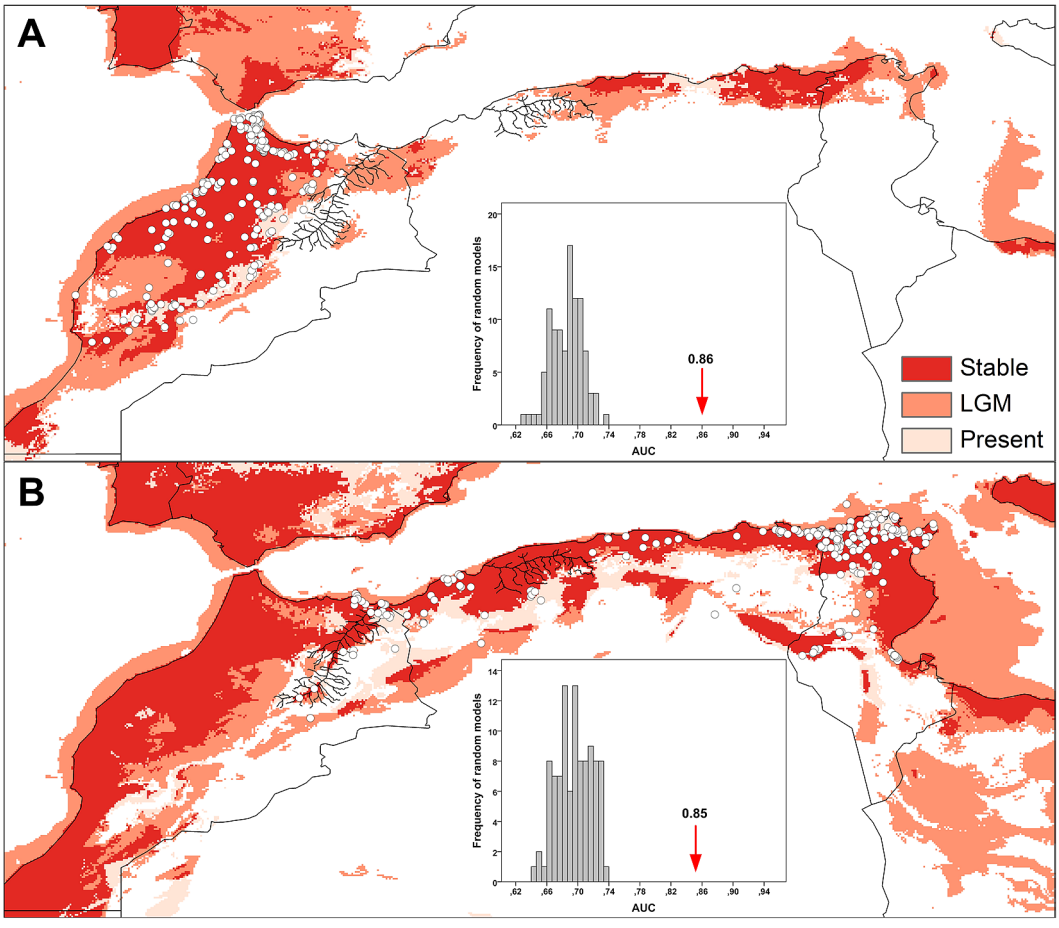

Figure 5. Potential species distribution models of *Discoglossus scovazzi* (A) and *D. pictus* (B) for the present and Last Glacial Maximum based on the TPT threshold. Results of null-models to test for significance of the SDMs and the available distribution data for both species are indicated (white dots). This figure is published in colour in the online version.

vazzi (BIO12 = 63.4%, BIO15 = 14.6%). The SDMs reveal climate stability in large parts of the species' distributions. The present and past SDMs for both *D. pictus* and *D. scovazzi* reveal large suitable areas in northern Africa extending well beyond the current distribution ranges of the species (fig. 5). The present SDMs for *D. scovazzi* indicate unsuitability of the Moulouya River Basin as well as in the Jbilets region north of Marrakech, whereas the present and past SDMs of *D. pictus* indicate a continued suitability in the Moulouya River Basin extending into most areas in Morocco, but excluding parts of the High Atlas.

The environmental space occupied by *D. pictus* and *D. scovazzi* as determined by PCA-env and ENFA is shown in Supplementary figs S10 and S11, respectively. Niche overlap between the species ($D = 0.452$) is limited. The niche equivalency hypothesis was rejected ($p = 0.02$), revealing significant differences between the two species' niches. The result of the randomization test of background similarity for PCA-env, however, shows that the two species' niches are significantly more similar than would be expected given the underlying environmental differences between the regions in which they occur (PCA-env, $p = 0.02$ and ENFA in a single direction, $p = 0.02$). These results are consistent with the large overlap predicted by the SDMs.

Discussion

We provide a first comprehensive assessment of the phylogeographic structure in *D. scovazzi*, given that the sole previous study (Zangari, Cimmaruta and Nascetti, 2006) included mtDNA sequences of only six individuals from two sites, and allozyme data of 28 individuals from four sites. The data suggest an overall weak phylogeographic structure of the species; only the populations from high elevations in the Atlas Mountains appear to be characterized by slightly divergent haplotypes, suggesting these populations originated from an ancestral population that diverged in a refugial area or in a sanctuary located in southern Morocco, possibly in the Atlas.

A pattern of generally low phylogeographic structure is also found in several other Moroccan amphibians, such as *Alytes maurus* Pasteur and Bons, 1962, *Amietophrynus mauritanicus* (Schlegel, 1841), *Bufotes boulengeri* (Lataste, 1879), and *Pelophylax saharicus* (Boulenger, 1913), all of which lack a clear phylogeographic structure in Morocco (Harris, Batista and Carretero, 2003; Batista et al., 2006; Stöck et al., 2008a; Harris and Perera, 2009; de Pous et al., 2013), whereas the treefrog *Hyla meridionalis* Boettger, 1874 is subdivided into three deep geographically structured mtDNA lineages in this country (Recuero et al., 2007; Stöck et al., 2008b).

While our data confirm the Moulouya River Basin largely coinciding with the range boundaries of *D. pictus* and *D. scovazzi*, the dense sampling herein also provided some surprising results. The area of Melilla and Nador appear to be populated by pure *D. pictus*, as we did not find any mitochondrial or nuclear alleles of *D. scovazzi* in the 17 specimens analyzed from this area. Hence, the species inventory of the Spanish territory of Melilla should include *D. pictus* rather than *D. scovazzi*. We cannot exclude that the presence of *D. pictus* in this area might be due to introduction. If however, the species is indeed native to this area, it provides evidence for the capacity of these frogs to cross big rivers, such as the Moulouya, even in the proximity of the estuary where the river is widest. Our study provides necessary data so that future efforts to characterize the contact zone between these two taxa can be focused more narrowly. Namely the area between Nador and Midar should be sampled in more detail to understand whether the two species might occur sympatrically at some sites within this region.

Although in this coastal area suitable habitat for *Discoglossus* does exist, it needs to be taken into account that large parts of the Moulouya River Basin further south are very arid and prob-

ably not suitable for these frogs, while for numerous arid-adapted taxa this valley has served as a dispersal corridor to the north (Bons and Geniez, 1996). The barrier effect for *Discoglossus* might be caused by the aridity of the valley rather than by the river itself. Given that *D. scovazzi* and *D. pictus* are not sister species (Pabijan et al., 2012), it is unlikely that the Moulouya River Basin played a role as a barrier triggering their primary vicariant divergence; more likely, this basin acts as a secondary barrier.

The present and LGM-SDMs of *D. pictus* and *D. scovazzi* show a large potential distribution in northern Africa extending well beyond the current known distribution ranges. Interestingly, the models predict that only *D. pictus* occurs in the Moulouya River Basin and that *D. scovazzi* is limited to the mountainous and wetter areas to the west. Also, in general, the area currently occupied by *D. scovazzi* appears to be fully suitable for *D. pictus* (except for high areas in the Atlas) while suitable areas for *D. scovazzi* within the *D. pictus* range are patchy. The LGM models show that the Moulouya River Basin remained largely unsuitable for *D. scovazzi*, while the extent of suitability for *D. pictus* increased in comparison with the present. The potential distribution during the LGM is larger than the present for both species. This is in agreement with previous studies (de Pous et al., 2011, 2013) and likely results from wetter and cooler annual climatic conditions in North Africa (Rognon, 1987; Wengler and Vernet, 1992). In northern Africa, *D. pictus* and *D. scovazzi* inhabit largely similar habitats with the exception that the former occurs in much drier areas and the latter occurs at high altitudes (up to 2650 m a.s.l.) in the Atlas Mountains. These environmental conditions are absent from the historical ranges of the other species respectively and this has likely contributed to rejection of the niche equivalency hypothesis. Both the SDMs and the niche overlap tests indicate that the niches of *Discoglossus* in northern Africa are conserved. The SDMs indicate the potential existence of a contact zone in several regions west of the Moulouya River Basin and this requires additional fieldwork in these areas.

Combining data from distribution ranges (*D. pictus* extending west of the Moulouya River Basin) and SDMs (large parts of the Moulouya valley and of the *D. scovazzi* range suitable for *D. pictus*, but less so vice-versa) suggests a scenario in which *D. scovazzi* had a historical range largely restricted to Morocco, possibly limited by the Moulouya River Basin, while *D. pictus* expanded its range westwards and crossed the Moulouya River near its estuary, leading to the currently observed contact zone largely coinciding with the Moulouya River Basin.

While the distribution of species of *Discoglossus* is predominantly allopatric, in three cases the distribution areas of species or major lineages of the genus overlap or abut. The first case is in Corsica, where the two species *D. montalentii* and *D. sardus* are broadly sympatric (Lanza et al., 1984, 1986; Zangari, Cimmaruta and Nascetti, 2006). *Discoglossus montalentii* mainly inhabits montainuous areas and breeds inside puddles of fast-flowing streams, while *D. sardus* is distributed mainly in temporary waters in the lowlands. Yet, several sites of close syntopy exist (Lanza et al., 1984, 1986; Vences, Glaw and Hirschberger, 1996) and so far, no indication of hybridization has been published. In this case, the taxa concerned are phylogenetically distant (Lanza et al., 1984, 1986; Fromhage, Vences and Veith, 2004; Pabijan et al., 2012; Biton et al., 2013), and differentiated both morphologically (Lanza et al., 1984; Clarke and Lanza, 1990; Capula and Corti, 1993; Clarke, 2007) and bioacoustically (Glaw and Vences, 1991). The other two cases are the contact zones which herein are characterized geographically in some detail. The ranges of *D. pictus* and *D. scovazzi* in Morocco appear to be characterized by a sharp boundary, without broad overlap. Although we cannot exclude isolated sympatric occurrence of the two taxa, it seems clear that such instances will be exceptional. The concordance between mitochondrial and nuclear markers in

our analysis suggests that instances of ongoing gene flow between these taxa are probably rare if they even exist. These two species are morphologically and bioacoustically similar (Clarke and Lanza, 1990; Capula and Corti, 1993; Vences and Glaw, 1996; Clarke, 2007) but do not seem to be sister taxa (Pabijan et al., 2012), and show a moderate genetic differentiation. The third example is the contact zone between two main Iberian lineages, which are considered distinct species by some (e.g., Busack, 1986; García-París and Jockusch, 1999; Martinez-Solano, 2004; Real et al., 2005) and subspecies by others (Lanza et al., 1986; Vences and Glaw, 1996; Zangari, Cimmaruta and Nascetti, 2006; Speybroeck, Beukema and Crochet, 2010; Pabijan et al., 2012; Vences, 2012; Sillero et al., 2014). These two mitochondrial lineages clearly are sister to each other (Pabijan et al., 2012); they show no consistent divergence in the nuclear markers investigated so far, and only comparatively weak divergence in mtDNA (e.g., Zangari, Cimmaruta and Nascetti, 2006; Velo-Antón, Martínez-Solano and García-Paris, 2008; Pabijan et al., 2012; data herein). Their uncorrected pairwise divergence in the 16S rRNA gene (as sequenced for instance by Fromhage et al., 2004) is only about 2%, and thus below the minimum threshold of 3% that characterizes many well-differentiated species of amphibians from their closest relatives (e.g., Fouquet et al., 2007). Although they might show weak morphological differentiation (Capula and Corti, 1993), it is uncertain whether this variation is clinal. Bioacoustically, no obvious differences have been observed (Vences and Glaw, 1996). In this example, mtDNA suggests a somewhat broader contact zone with syntopic occurrence of the two lineages in at least some locations, and either incomplete lineage sorting or extensive gene flow in the nuclear genes taken into account to date.

In a nutshell, we here propose the hypothesis that these three examples might represent different stages of the speciation and differentiation process: *D. montalentii* and *D. sardus* are strongly divergent genetically, and also have diverged in morphology, ecology and bioacoustics, allowing them to occur in sympatry and sometimes in close syntopy, without apparent admixture. *Discoglossus pictus* and *D. scovazzi* are less divergent genetically and do not differ clearly in morphology, ecology and bioacoustics, yet the genetic divergence might be strong enough to avoid admixture across their contact zone in eastern Morocco. Finally, the two Iberian lineages show the weakest divergence from a mitochondrial perspective and extensive sharing of nuclear alleles. These findings agree with considering *jeanneae* as subspecies of *D. galganoi* following the rationale of Speybroeck, Beukema and Crochet (2010) at least until more detailed analysis of the contact zone using highly variable nuclear markers may indicate absence of gene flow among them.

This study mainly aimed at characterizing the geographical ranges of *Discoglossus* lineages in Iberia and Morocco, and providing a first asssessment of the phylogeographic differentiation of *D. scovazzi*. It is outside our scope to comprehensively analyze and understand the evolutionary and molecular dynamics in the contact zones of these frogs, and the limited molecular data provided herein can only help defining hypotheses that will require future testing. We propose that the double peaks observed in the chromatograms of a large number of individuals are related to the admixture of *jeanneae* and *galganoi* in their contact zone. Whether these additional copies are all nuclear pseudocopies (NUMTs) or are partly caused by heteroplasmy can only be inferred by more extensive molecular work. In two individuals, we could show that they indeed contain COB variants of both *jeanneae* and *galganoi*, which may have resulted from hybridization and admixture between these two taxa. However, by direct sequencing and phasing, the two sequences per individual always clustered with the same lineage (either both with *galganoi*, or both with *jeanneae*), suggesting that one of the copies present in the respective individuals

might occur more commonly or amplify preferentially. Whether all double-peak individuals bear a combination of *jeanneae* and *galganoi* sequences is therefore completely unclear; in some cases, double peaks are only at single or few positions which do not constitute diagnostic differences between the two lineages. Altogether this suggests that the use of mtDNA as marker for a clear-cut distinction of *jeanneae* and *galganoi* might not always yield reliable results. Future studies of this phenomenon could benefit from next-generation sequencing methods to identify all underlying sequences present in amplicons. Similar situations have been found in other taxa, including bristletails (Baldo et al., 2011) and lizards (Podnar et al., 2007; Miraldo et al., 2012). In all these cases, hybridization between species or lineages led to the presence of more than one mitochondrial genome in the individuals concerned. Usually, the variation was located in NUMTs, but low levels of heteroplasmy were also detected (Miraldo et al., 2012). It is a fascinating perspective for future studies to test whether selective advantages of particular mitochondrial variants, and/or genomic conflict might be responsible for these bizarre patterns, and whether such genomic conflict might aid in characterizing the divergence process, or absence thereof, between the lineages involved (see Crespi and Nosil, 2013). Our study suggests that the contact zone of *D. g. galganoi* and *D. g. jeanneae* might be a suitable model for such future studies.

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