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β-fibrinogen intron 7 variation in Discoglossus (Anura: Discoglossidae): implications for the taxonomic assessment of morphologically cryptic species

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β-fibrinogen intron 7 variation in *Discoglossus* (Anura: Discoglossidae): implications for the taxonomic assessment of morphologically cryptic species

Guillermo Velo-Antón[1,*], Iñigo Martínez-Solano[2], Mario García-París[3]

Abstract. The generalized use of nuclear introns in combination with mitochondrial DNA data in molecular systematic and intraspecific phylogeographical studies is providing new insights into the complex evolutionary histories of taxa surviving the Quaternary glaciations. Previous studies have highlighted the suitability of the beta-fibrinogen intron 7 (*β-fibint7*) for phylogenetic and phylogeographic studies in a wide variety of taxa, including amphibians. Here we use sequences of this marker to assess inter- and intraspecific variation in *Discoglossus* (Discoglossidae), with special emphasis on geographic patterns of genetic structure in the Iberian Peninsula, where recent studies have questioned the taxonomic status of *D. jeanneae*. We obtained 81 sequences of *β-fibint7* from samples including all currently recognized species except *D. montalentii* and 37 populations in the Iberian Peninsula and compared levels of genetic variation with those observed in a fragment of similar length of the mtDNA gene cytochrome b. The sequence of *β-fibint7* in *Discoglossus* is the shortest described for amphibians so far, 378 base pairs. In general, we found low levels of variability (only 26 parsimony-informative sites in the dataset), with no alternatively fixed haplotypes in samples attributed to *D. galganoi* or *D. jeanneae* based on their mtDNA. Values of pairwise sequence divergence between non-Iberian species ranged from 1.1% to 4.5% (13.3% to 20.9% in mtDNA). The patterns observed in samples from the Iberian Peninsula are consistent with either incomplete lineage sorting or ongoing gene flow between *D. galganoi* and *D. jeanneae.* We conclude by reviewing the genetic evidence available to address the taxonomic status of Iberian species of *Discoglossus*.

Keywords: beta-fibrinogen intron 7, *Discoglossus*, Iberian Peninsula, mtDNA, nuclear intron, systematics.

Introduction

The use of sequence data from nuclear introns in molecular systematics and in phylogeographic studies has become increasingly prevalent, mostly in combination with mitochondrial DNA (mtDNA) data (Dolman and Moritz, 2006; Heckman et al., 2007; Leavitt et al., 2007). In those cases, the analyses of several potentially unlinked molecular markers, with very different inheritance profiles and mutation rates across a comprehensive geographic set of samples is crucial to bridging the gap between gene and species trees (Maddison, 1997; Templeton, 2004). Combining information from nuclear and mtDNA markers thus constitutes a powerful tool to infer complex evolutionary histories, like those exhibited by species that survived the glaciations of the Quaternary in allopatric refugia and underwent several successive cycles of isolation (retreat) and admixture (expansion) following climatic changes (Knowles and Carstens, 2007).

Amongst the increasing list of introns used in molecular systematics, one of the most common is the beta-fibrinogen intron 7 (*β-fibint7*). The availability of nearly-universal primers and its relatively fast evolutionary rate have proven useful in recovering phylogenetic relationships in a variety of vertebrate taxa, from amphibians to birds and mammals (Prychitko and Moore, 1997; Weibel and Moore, 2002; Yu and Zhang, 2005; Sequeira et al., 2006; Gonçalves et al., 2007). In some cases, high levels of intraspecific variation have allowed characterization of phy-

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logeographic patterns, sometimes corresponding closely to those observed at the mtDNA level (Godinho et al., 2006).

Discoglossus and its close fossil relatives conform one of the most ancient clades of frogs, with an independent evolutionary history since the Lower Cretaceous (Sanchiz, 1998; San Mauro et al., 2004), and constitute an interesting group to further evaluate the potential utility of this marker. The genus includes six extant species endemic to the western Mediterranean Basin that have been widely studied from morphological, molecular and ecological perspectives (Capula et al., 1985; Glaw and Vences, 1991; Capula and Corti, 1993; Vences and Glaw, 1996; Fromhage et al., 2004; Zangari et al., 2006). *Discoglossus galganoi* and *D. jeanneae* are endemic to the Iberian Peninsula; *D. scovazzi* is present in north Africa west of the Moulouya River; *D. pictus auritus* is also present in north Africa east of the Moulouya River, with introduced populations in NE Iberia and south-eastern France; *D. pictus pictus* inhabits Sicily, Malta and Gozo; *D. montalentii* is found in Corsica and finally, *D. sardus* is found in Corsica, Sardinia and smaller islands in the Tyrrhenian Sea. Whereas phylogenetic relationships between taxa have been well resolved (Fromhage et al., 2004; Zangari et al., 2006), important questions remain unresolved or controversial, most notably the taxonomic status of the Iberian endemic *D. jeanneae* Busack, 1986. The most recent and comprehensive molecular dataset published so far (Zangari et al., 2006) found little genetic differentiation in allozymes between populations assigned to this species and its sister taxon, *D. galganoi*, also endemic to the Iberian Peninsula, and suggested recognition as subspecies (*D. galganoi galganoi* and *D. g. jeanneae*) as the most appropriate taxonomic arrangement for the two Iberian taxa. However, despite the low genetic distances, both Iberian species were recovered as monophyletic, implying some level of nuclear differentiation.

Our aim in this study is to evaluate the phylogenetic and phylogeographic utility of *β-fibint7* in *Discoglossus*, with special attention to geographic patterns of variation in the Iberian Peninsula. We compare our results with published and new mtDNA sequences from the same individuals used in this study and discuss the genetic evidence available to address the taxonomic status of *D. jeanneae*.

Materials and methods

Sampling

We obtained tissue samples from all extant species of *Discoglossus* except *D. montalentii*. The final dataset included a total of 68 samples from Iberian *Discoglossus* (*D. galganoi* and *D. jeanneae*) collected at 37 localities across the Iberian Peninsula, three samples of *D. scovazzi* from one locality in Morocco, two samples of *D. pictus* from two localities in Tunisia, and eight samples of *D. sardus* from Corsica and Sardinia (see table 1, fig. 1).

DNA amplification and alignment

Total genomic DNA was extracted from ethanol-preserved tissues using a standard proteinase K/phenol chloroform extraction protocol. A total of ~510 base pairs (bp) corresponding to beta-fibrinogen intron 7 and part of the exon flanking regions were amplified via polymerase chain reaction (PCR) using the primers FIB-B7U and FIB-B7L (Prychitko and Moore, 1997) in all 81 samples used in this study. PCR reactions consisted of 38 cycles with a denaturing temperature of 94°C (1 min), annealing at 50°C (1 min) and extension at 72°C (1 min). PCR reactions were performed in a total volume of 13 μl with 30-60 ng of template DNA, including 0.15 μl of Taq polymerase (Biotools, 5 U/ml), 0.5 μl of each primer (10 mmol/l), 0.5 μl of dNTPs (10 mmol/l), 0.7 μl of $MgCl_2$ (25 mmol/l) and 1.25 μl of reaction buffer (Biotools, Tris-HCl, pH = 8.3). Double-strand templates were cleaned using sodium acetate and ethanol to precipitate the PCR products and then re-suspended in 20-25 μl of ddH_2O. Sequencing reactions were performed for both strands and sequenced on an ABI PRISM 3700 DNA sequencer following the manufacturer's instructions.

For comparisons, we compiled a mtDNA dataset (70 individuals) including most of the samples sequenced for *β-fibint7*. This dataset is composed of previously published partial sequences of the cytochrome b (*cob*) gene (Martínez-Solano, 2004), complemented with 24 new sequences. Details of amplification and sequencing of *cob* are provided in Martínez-Solano (2004). Sequences of *D. montalentii* were downloaded from GenBank (Accession Numbers: AY347430 and AY347431) and included in the final *cob* alignment for reference.

Sequences were compiled, edited and aligned manually with ProSeq v.2.91 (Filatov, 2002). Heterozygous nucleotide positions were identified as double peaks in the

Table 1. Sampling localities, including latitude and longitude coordinates, voucher numbers, sampling sizes and haplotypes observed in *β-fibint7* and *cob* datasets. Region numbers refer to population groups in fig. 1.

Species	Country	Region	Locality	Vouchers	*b-fibint7*	*cob*	Latitude	Longitude	b-*fibint7* haplotype	*cob* haplotype
D. galganoi	Portugal	1	Cernache de Bonjardim	IMS 338, 339	2	2	39°48′36″N	8°10′33″W	I, V, VII	VI
		2	Vagos	IMS 324, 325	2	2	40°32′24″N	8°32′00″W	I	I, IV
		3	Póvoa do Varzim	IMS 373, 374	2	2	41°22′60″N	8°46′00″W	III, VIII	I, V
	Spain	4	Pontevedra: Isla de Ons	ONS 1, 2	2	2	42°21′55″N	8°56′17″W	I, III	I
		5	Coruña: Torre de Hercules	Coruña 1, 2	2	2	43°23′14″N	8°24′9″W	I, III	II
		6	Lugo: Gomeán	IMS 215, 228, 229	3	2	42°56′06″N	7°24′18″W	I, III	II
		7	Asturias: Tineo	IMS 218, 219, 220, 222	4	3	43°20′18″N	6°24′49″W	I, III	I
		8	León: Reliegos	IMS 4, 6	2	2	42°28′35″N	5°21′17″W	I, II, III	I, III
		9	Zamora: Codesal	IMS 230	1	2	41°58′15″N	6°22′54″W	I, II	I
		10	Burgos: Quintanilla-Escalada	IMS 51, 52	2	2	42°47′52″N	3°46′18″W	I, II	I
		13	Madrid: Soto del Real	IMS 34	1	1	40°45′57″N	3°46′10″W	I, X	VI
		13	Madrid: Cenicientos	IMS 17	1	1	40°15′14″N	4°29′57″W	II	VI
		13	Madrid: Navalagamella	IMS 60	1	1	40°28′00″N	4°07′00″W	I, II	VI
		13	Madrid: Villamantilla	IMS 47, 48	2	2	40°22′21″N	4°08′11″W	I, II	VI
		13	Madrid: Cerceda	IMS 540	1	1	40°42′00″N	3°55′60″W	II, X	VI
		14	Ávila: Monbeltrán	IMS 42	1	1	40°15′30″N	5°01′12″W	I	VI
		15	Toledo: El Real de San Vicente	IMS 234, 235	2	2	40°08′20″N	4°41′31″W	I, II	VI
		17	Badajoz: Mérida	IMS 217, 232, 233	3	3	38°57′37″N	6°21′36″W	I, II, VI, IX	VI
		18	Ciudad Real: Poblete	IMS 44, 45, 46	3	2	38°56′19″N	3°58′55″W	I, II	VIII
		22	Sevilla: El Ronquillo	IMS 32	1	1	37°42′05″N	6°06′57″W	VI, X	VII
		23	Huelva: Rosal de la Frontera	IMS 533, 534	2	1	37°58′00″N	7°13′00″W	III, IV, XIII	VI

Table 1. (Continued).

Species	Country	Region	Locality	Vouchers	*b-fibint7*	*cob*	Latitude	Longitude	b-*fibint7* haplotype	*cob* haplotype
D. jeanneae	Spain	10	Burgos: Entrambosríos	IMS 49, 50	2	2	43°02′53″N	3°42′02″W	I, II	IX
		11	Zaragoza: Romanos	IMS 53	1	1	41°07′40″N	1°16′36″W	II	IX
		12	Guadalajara: Mondéjar	IMS 26	1	1	40°19′53″N	3°06′28″W	I, II	IX
		12	Guadalajara: Driebes	IMS 27	1	1	40°14′55″N	3°02′41″W	I, II	IX
		12	Guadalajara: Yebes	IMS 62	1	1	40°50′55″N	3°28′01″W	I, II	IX
		12	Guadalajara: Uceda	IMS 63	1	1	40°07′38″N	3°12′12″W	II	IX
		13	Madrid: Aoslos	IMS 1, 2, 10, 11, 12	5	4	41°03′15″N	3°36′08″W	I,II,X	IX, X
		13	Madrid: Valdilecha	IMS 22	1	1	40°16′60″N	3°17′60″W	II	IX
		13	Madrid: Belmonte de Tajo	IMS 58	1	1	40°08′48″N	3°25′37″W	II	IX
		13	Madrid: Chinchón	IMS 59	1	1	40°07′60″N	3°25′00″W	I, II	IX
		16	Cuenca: Belinchón	IMS 73	1	2	40°03′05″N	3°02′51″W	I	IX
		19	Albacete: Ojos de Villaverde	GVA135	1	1	38°48′51″N	2°22′06″W	VI	XI
		20	Jaén: Iznatoraf	IMS 250, 251, 252	3	2	38°08′60″N	3°01′60″W	I, VI, XI	IX
		20	Jaén: Santuario Nuestra Señora Cabeza	IMS 253, 254, 255, 256	4	4	38°05′59″N	4°01′04″W	II, XI	IX
		21	Málaga: Fuengirola	IMS 257	1	1	36°31′60″N	4°37′00″W	I	IX
		24	Cádiz: San José del Valle	IMS 33, 36, 37	3	1	36°36′23″N	5°24′20″W	I, II, IX, XII	IX
D. scovazzi	Morocco		Ifrane	IMS 18, 19, 28	3	2	33°31′60″N	5°06′00″W	XIV, XV, XVI	XII, XIII
D. pictus	Tunisia		Gafsa Oasis Sud	GVA 301	1	1	34°23′18″N	8°47′50″E	XVII, XVIII	XIV
			Monastir	GVA 302	1	1	35°45′33″N	10°48′49″E	XIX, XX	XV
D. sardus	France		Corsica	GVA 73, 74, 75, 76, 79, 81	6	4	41°39′32″N	9°11′23″E	XXI, XXII	XVI, XVII, XVIII
	Italy		Sardinia	GVA 85, 86	2	2	40°59′21″N	9°12′37″E	XXI, XXII	XVI
D. montalentii	France		Corsica		0	2				XIX, XX
Total					81	74				

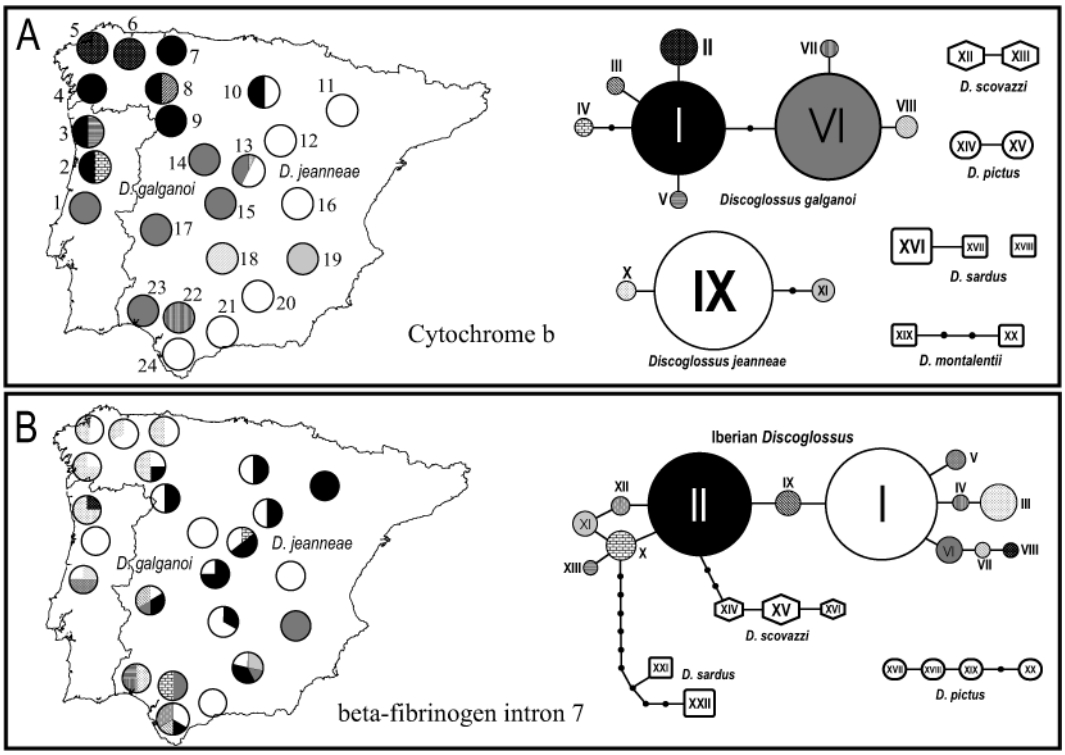

Figure 1. Sampling locations of *D. galganoi* and *D. jeanneae* in the Iberian Peninsula. Pie charts on maps represent sampling localities (pooled based on geographic proximity, see "Regions" in table 1) and haplotype frequencies for *cob* (1A) and *β-fibint7* sequences (1B). On the right, statistical parsimony networks depicting relationships between haplotypes, with sizes proportional to their frequencies. Numbers in fig. 1A represent population groups as listed in table 1.

electropherograms (Brumfield et al., 2003) and coded according to standard IUPAC ambiguity codes. All sequences obtained for this study were deposited in GenBank (Accession numbers EU744884-EU744911).

Genetic analyses

We used the Bayesian approach implemented in PHASE version 2.1 (Stephens et al., 2001) to reconstruct haplotypes for heterozygous individuals. Only haplotypes with posterior probabilities (pp) over 0.90 were included in the analyses.

Haplotype networks for both nuclear and mitochondrial datasets were constructed and compared using the statistical parsimony algorithm implemented in TCS version 1.21 (Clement et al., 2000) treating indels (regardless of their size) as single mutational events (fig. 1). Intra- and interspecific corrected (Kimura-2 parameter) genetic distances between samples were estimated with the software package MEGA 3.1 (Kumar et al., 2004).

Two approaches were used to determine the presence of recombination in the nuclear gene. First, the minimum number of recombination events (Rm) (Hudson and Kaplan, 1985) was determined using DNASP version 4.10.9 (Rozas et al., 2003). Additionally, the Phi test implemented in SplitsTree4 (Huson and Bryant, 2006) was used to calculate the pairwise homoplasy index (Φ_w) of Bruen et al. (2006) to test for recombination. The ability of this statistic to distinguish recurrent mutation from recombination has been demonstrated through simulations (Bruen et al., 2006).

In order to test the phylogenetic potential of the markers used we performed standard phylogenetic analyses on both nuclear and mtDNA datasets. These included Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian analyses. MP and ML analyses were performed with PAUP* 4.0b10 (Swofford, 2003). First, we analyzed the data to determine the best fitting substitution model for the maximum likelihood and Bayesian analyses, which was selected with the Akaike information Criterion (AIC) as implemented in ModelTest 3.7 (Posada and Crandall, 1998). For MP and ML analyses the heuristic search algorithm was performed with 10 random additions of sequences and tree-bisection-reconnection (TBR) branch swapping. Statistical support for the resulting topologies was assessed using nonparametric bootstrap (Felsenstein, 1985), with 1000 pseudorreplicates for MP and 100 for ML. Bayesian analyses were carried out with Mr. Bayes v3.1 (Ronquist and Huelsenbeck, 2003), defining three partitions corresponding to 1st + 2nd + 3rd codon positions in the *cob* dataset. The *β-fibint7* alignment was analyzed as an unpartitioned dataset. Analyses were run for 10^6 generations with a sampling frequency of 10^4 generations. Of the resulting 1000

trees, the first 250 trees were discarded as burn in after checking for stationarity with TRACER v1.3 (Rambaut and Drummond, 2004).

Results

Nuclear and mitochondrial genetic diversity

The final *β-fibint7* alignment contained 81 sequences. Exon flanking regions were identified by comparison of the sequences obtained with those deposited in GenBank (∼90% similarity with sequences of diverse species of rodents, primates and birds) and excluded from further analyses due to the low levels of variability (only 2 single-site mutations in the ingroup). After excluding the flanking exon regions, the aligned sequence length was only 378 bp long. A + T content was high (∼70%), as reported in previous studies using this marker (Prychitko and Moore, 1997; Godinho et al., 2005; Yu and Zhang, 2005; Sequeira et al., 2006; Gonçalves et al., 2007). There were only 30 variable sites in all samples analyzed (26 parsimony-informative sites), with two indels: a 3 bp insertion in *D. scovazzi* and a 5 bp deletion in Iberian samples. COLLAPSE 1.2 (D. Posada, http://darwin.uvigo.es) reduced the nuclear dataset to 22 haplotypes for subsequent analyses.

For *cob*, 74 sequences 355 bp long were compiled. There were 107 variable positions (104 parsimony-informative sites) defining 20 haplotypes. No insertions, deletions, or stop codons were observed, and base content was typical of mtDNA, suggesting the sequences obtained are functional mtDNA copies rather than nuclear pseudogenes.

Genetic differentiation

The comparison of nuclear and mtDNA results revealed important differences between datasets. First, despite clear sorting of Iberian samples into two highly divergent haplogroups based on mtDNA, in accordance with previous studies (García-París and Jockusch, 1999; Fromhage et al., 2004; Martínez-Solano, 2004; Real et al., 2005; Zangari et al., 2006), there were no fixed haplotypes in the nuclear dataset corresponding to *D. galganoi* and *D. jeanneae* (fig. 1). The second difference refers to lower values of genetic divergence in the nuclear dataset (table 2). For instance, interspecific divergence values were higher for mtDNA than nDNA sequences, with K2p-corrected genetic distances ranging from 8.6% (*D. galganoi* − *D. jeanneae*) to 21.7% (*D. galganoi* − *D. pictus*) for *cob* and from 1.1% (*D. galganoi/D. jeanneae* − *D. scovazzi*) to 4.5% (*D. pictus* − *D. sardus*) for *β-fibint7* (table 2).

Two independent haplotype networks were recovered under the 95% statistical parsimony criterion in the nuclear dataset, one corresponding to samples of *D. pictus* and the other to *D. sardus*, *D. scovazzi* and the Iberian samples (fig. 1B). The number of mutational steps separating the *D. pictus* network from that including Iberian *Discoglossus*, *D. scovazzi* and *D. sardus* were 11, 12 and 20 respectively.

The Iberian samples are represented by 13 haplotypes in the main network, with two of

Table 2. K2p-corrected distances between *Discoglossus* species. Nuclear (*β*-fibrinogen intron 7) genetic distances are shown above the diagonal; mitochondrial (cytochrome b) distances below the diagonal. On the diagonal (boldface), K2p-corrected distances of combined Iberian samples (*D. galganoi* + *D. jeanneae*) with respect to other species are shown (left: mtDNA; right: nDNA).

	D. jeanneae	*D. galganoi*	*D. scovazzi*	*D. sardus*	*D. pictus*	*D. montalentii*
D. jeanneae	**–/0.008**	–	0.011	0.027	0.033	–
D. galganoi	0.086	**–/0.008**	0.011	0.027	0.033	–
D. scovazzi	0.128	0.135	**0.007/0.004**	0.028	0.036	–
D. sardus	0.163	0.172	0.142	**0.020/0.011**	0.045	–
D. pictus	0.188	0.217	0.165	0.168	**0.007/0.010**	–
D. montalentii	0.168	0.206	0.166	0.201	0.206	**0.0075/–**

them (I and II) found in high frequencies and connected by an intermediate haplotype found in lower frequencies in southern and central populations (IX, see table 1 and fig. 1B). Both common haplotypes are widely distributed in the Iberian Peninsula, although haplotype I was more commonly found in western populations and haplotype II in eastern populations. All other haplotypes are closely related to either one of these two most common haplotypes. Haplotype III was the most common and widely distributed in northwestern Iberia, whereas other haplotypes show a limited geographic distribution, perhaps a consequence of sample size. In general, haplotype diversity is higher in southern populations (fig. 1B). The minimum number of recombination events detected by DNASP was 3 (Rm = 3). However, the Φ_w test did not find statistically significant evidence for recombination ($P = 0.36$) suggesting recurrent mutation rather than recombination in the *β-fibint7* sequences.

With respect to the mtDNA dataset, seven independent networks were recovered, each one corresponding to one of the six species of *Discoglossus* except *D. sardus*, whose haplotypes did not group together but formed two independent networks (fig. 1A). Iberian samples are separated into two independent networks, corresponding to *D. galganoi* (8 haplotypes) and *D. jeanneae* (3 haplotypes, fig. 1A). As shown by previous studies, genetic diversity is much lower in the latter, with a very common and widespread haplotype (IX) and two more restricted haplotypes: X and XI. In contraposition to the pattern observed in nuclear DNA, most variation was found in the northwest of the Iberian Peninsula.

Due to the low number of informative sites in the *β-fibint7* dataset, the trees recovered in all analyses only support the monophyly of *D. sardus, D. scovazzi* and *D. pictus*. The Iberian haplotypes are not recovered as a monophyletic group, and they cluster with haplotypes found in *D. scovazii* and *D. sardus* (fig. 2). The only molecular evidence supporting monophyly of Iberian haplotypes is the presence of a 5 base pair deletion observed in all Iberian samples analyzed. In contrast, the analysis of the mtDNA dataset produced well-resolved topologies, with high support values for the monophyly of all species studied as well as for some of their interrelationships (fig. 2). Thus, *D. galganoi* and *D. jeanneae* are sister to each other and in turn sister to *D. scovazzi* (although this relationship is not supported in MP analyses). No other relationships were supported in our analyses.

Discussion

The utility of the beta-fibrinogen intron 7 for phylogenetic and phylogeographic studies in *Discoglossus* is limited by its small size (378 bp, the shortest described in amphibians so far) and low genetic variability (only 30 variable sites in our dataset), resulting in few informative characters. This contrasts with the results of previous studies using this marker in amphibians. For instance, Sequeira et al. (2006) report intron sizes ranging from 400 bp in *Pleurodeles waltl* to 1123 bp in *Salamandra atra*; they were able to obtain good resolution in phylogenetic analyses within Salamandridae. Additionally, Gonçalves et al. (2007) found significant levels of variation at both intra- and interspecific levels in the 634 bp-long sequences of midwife toads (*Alytes*) analyzed in their study. Pairwise Kimura-2 parameter-corrected genetic distances between species pairs in *Alytes* are 2.5 to 7.6 times higher in mtDNA (*cob*, data from Martínez-Solano et al., 2004) than in *β-fibint7* (Gonçalves et al., 2007), and the corresponding values in *Discoglossus* are similar, 3.7-5 times higher in mtDNA than in *β-fibint7* (this study).

Corrected *β-fibint7* pairwise genetic distances between species of *Discoglossus* range from 1.1% to 4.5%. These levels of variation allow species discrimination, with the exception of samples from endemic Iberian *Discoglossus* and are higher than those observed for other nuclear markers, like *RAG-1*. Based on comparison of three 556 bp sequences

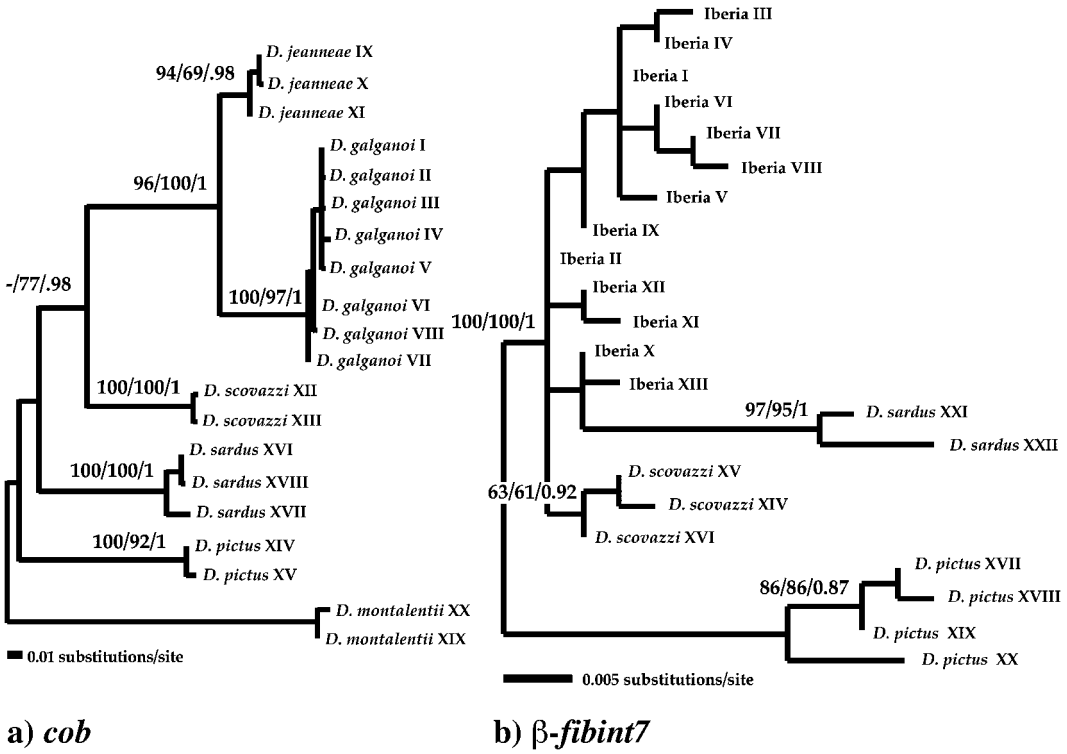

Figure 2. Maximum likelihood trees of *cob* (a) and *β-fibint7* (b) haplotypes in *Discoglossus.* Support values on branches refer to Maximum Parsimony, Maximum Likelihood and Bayesian Posterior Probabilities, respectively.

from three different *Discoglossus* species downloaded from GenBank (Accession Numbers: AY323757, AY583338 and AY364202), pairwise uncorrected genetic distances in *RAG-1* are only 0.36% between *D. sardus* and *D. galganoi* and 0.71% between *D. pictus auritus* and *D. sardus/D. galganoi.* Thus, whereas levels of variation are appropriate for phylogenetic analyses, the low number of informative characters provided by *β-fibint7* forces its use in combination with other markers.

No alternatively fixed *β-fibint7* haplotypes corresponding to populations identified as *D. galganoi* or *D. jeanneae* based on their mtDNA were found in our study. This contrasts with the sharp genetic break in mtDNA (8.6% corrected pairwise distances), similar to that documented previously (García-París and Jockusch, 1999; Fromhage et al., 2004; Martínez-Solano, 2004; Zangari et al., 2006), but is consistent with the low levels of genetic differentiation at allozyme loci between *D. galganoi* and *D. jeanneae* reported by Zangari et al. (2006) (mean $D_{Nei} = 0.05$). Two potential explanations for this discordance can be proposed: 1) incomplete sorting of ancestral polymorphisms; and 2) male-biased gene flow across mtDNA contact zones. A special case of the second explanation would involve range expansion and contraction associated with climatic oscillations: each time the ranges expanded and the two populations came into contact there would be admixture, whereas each time the ranges contracted, drift would cause mtDNA to fix different haplotypes in different refugia, but the same would not happen in nuclear genes due to their higher effective population sizes. At present, there is no evidence from field studies supporting or rejecting the existence of male-biased dispersal in *Discoglossus.* As indicated by García-París and Jockusch (1999), in *Discoglossus* females are the heterogametic sex, and according to Hal-

dane's rule, hybrid females may be negatively selected, resulting in spread of nuclear genes via hybridization while mtDNA haplotypes remain stationary. With respect to the first alternative, distinguishing between incomplete lineage sorting and gene flow remains a challenging task in molecular systematics, although new methodological tools are now available (Hey, 2006). Unfortunately, the low number of variable characters in the *β-fibint7* dataset was insufficient to estimate levels of gene flow from our dataset under the Isolation with Migration model implemented in IMa (Hey and Nielsen, 2007). Despite using different combinations of starting values for the different parameters in the model, the results consistently produced flat likelihood surfaces for some of these parameters, reflecting a lack of informative characters in our dataset (data not shown). A different, qualitative approach to distinguishing patterns caused by gene flow from those produced by incomplete lineage sorting is to analyze geographic patterns of haplotype distribution, as incomplete lineage sorting will tend to produce random associations between haplotypes and geography whereas, if gene flow is responsible for the pattern observed, introgressed haplotypes will be located in the vicinities of mtDNA contact zones. Based on this criterion (but taking into account limitations regarding low numbers of segregating sites) incomplete lineage sorting is certainly a possible explanation for our results. However, haplotypes I and II are most commonly found in western and eastern populations, respectively, suggesting some genetic structuring (see fig. 1B). The possibility of incomplete lineage sorting was also considered by Zangari et al. (2006) when discussing the results of their allozyme and mtDNA study, although they favoured an evolutionary scenario where post-Pliocene spread of genes across contact zones produced the observed genetic homogeneity at the nuclear level in Iberian *Discoglossus*. Ultimately, low genetic distances in nuclear markers led them to suggest subspecific status for the two Iberian species of *Discoglossus*. However, as they correctly point out, no detailed study of potential contact zones has been carried out, and incomplete lineage sorting certainly remains a plausible explanation of both our and their results. This has important implications for the taxonomic status of *D. jeanneae* because in the absence of direct evidence of gene flow across mtDNA borders, considering specific or subspecific status for *D. jeanneae* depends exclusively of application of an arbitrary genetic threshold for species delineation. While genetic distances reported in Zangari et al. (2006) are indeed very small (mean $D_{Nei} = 0.05$), this value is based on the analysis of only four populations of each species. Our results suggest future studies should be focused on potential contact zones between the two species and use larger sample sizes and more variable nuclear markers (microsatellites). In the meantime, until more comprehensive analyses of contact zones are carried out that address in detail the issue of gene flow across mtDNA contact zones in Iberian *Discoglossus*, we call for caution in adopting taxonomic changes and suggest maintaining species status for *D. galganoi* and *D. jeanneae*, as currently reflected by conservation legislation in Spain.

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