Volume 24 (October 2014), 261–265

Herpetological Journal

SHORT NOTE

Published by the British Herpetological Society

Isolation and characterisation of novel polymorphic microsatellite loci in Iberian painted frogs (*Discoglossus galganoi* and *D. jeanneae*), with data on cross-species amplification in *Discoglossus* and *Latonia* (Alytidae)

Jorge Gutiérrez-Rodríguez[1], Daniele Salvi[2], Eli Geffen[3], Sarig Gafny[4] & Íñigo Martínez-Solano[2,5]

[1]*Museo Nacional de Ciencias Naturales, CSIC, c/ José Gutiérrez Abascal, 2, 28006 Madrid, Spain*

[2]*CIBIO, Research Centre in Biodiversity and Genetic Resources, InBIO, Universidade do Porto, Campus Agrário de Vairão, Rua Padre Armando Quintas, s/n, 4485-661 Vairão, Vila do Conde, Portugal*

[3]*Department of Zoology, Tel Aviv University, 69978 Tel Aviv, Israel*

[4]*School of Marine Sciences, Ruppin Academic Center, Michmoret 40297, Israel*

[5]*Instituto de Investigación en Recursos Cinegéticos (IREC), CSIC-UCLM-JCCM, Ronda de Toledo, s/n, 13071 Ciudad Real, Spain*

Mediterranean painted frogs (genus *Discoglossus* Otth, 1837) are distributed across western Europe, North Africa and some Mediterranean islands. Previous studies have focused on their phylogenetic relationships, but the taxonomic position of the Iberian taxa (*D. galganoi* and *D. jeanneae*) is still under debate. By using microsatellites, patterns and rates of admixture can be quantified. We report the characterisation of eighteen novel polymorphic microsatellite loci in Iberian painted frogs. These loci were also tested in all other species of *Discoglossus* and in the recently rediscovered and highly endangered relative *Latonia nigriventer*. Two to eleven loci amplified in these species, and the number of polymorphic loci ranged from zero (in *Latonia*) to eight (in *D. scovazzi*). The new markers will be useful in addressing questions related to the evolutionary history, population structure, and conservation of Iberian *Discoglossus*. They also have potential for use in the North African species *D. scovazzi* and *D. pictus*, the latter of which is an invasive species rapidly expanding its range in southeast France and northeast Spain.

Key words: amphibians, *Discoglossus*, Iberian Peninsula, *Latonia nigriventer*, microsatellites

Mediterranean painted frogs (genus *Discoglossus* Otth, 1837) belong to the family Alytidae, an ancient anuran clade originated in the Jurassic that currently contains a total of only 12 Palaearctic species in the subfamilies Discoglossinae (7) and Alytinae (5). The genus *Discoglossus* is distributed across Western Europe, North Africa and some Mediterranean islands. Currently, five or six species (depending on authors) are recognised within the genus: *Discoglossus montalentii* Lanza, Nascetti, Capula & Bullini, 1984 in Corsica; *D. sardus* Tschudi In Otth, 1837 in Sardinia and Corsica; *D. pictus* Otth, 1837 in Sicily, Malta, Tunisia and Algeria and introduced in SE France and Catalonia (NE Spain); *D. scovazzi* Camerano, 1878 in Morocco; *D. galganoi* Capula, Nascetti, Lanza, Bullini & Crespo, 1985 and *D. jeanneae* Busack, 1986 in the Iberian Peninsula. Until recently, the genus *Discoglossus* has been thought to be the only representative of the subfamily Discoglossinae. However, in 2011, the Hula painted frog from Israel *D. nigriventer* Mendelssohn & Steinitz, 1943 was re-discovered, and based on genetic and morphological data it was reassigned to the genus *Latonia*, which is a sister taxon to *Discoglossus* (Biton et al., 2013).

Phylogenetic relationships among species of *Discoglossus* are relatively well established (Fromhage et al., 2004; Zangari et al., 2006; Velo-Antón et al., 2008; Pabijan et al., 2012; Biton et al., 2013), but the taxonomic position of the Iberian taxa is still under debate. Some authors consider *D. jeanneae* as a subspecies of *D. galganoi* (Lanza et al., 1986; Capula & Corti, 1993; Vences & Glaw, 1996; Zangari et al., 2006; Pabijan et al., 2012), whereas others recognise it as a valid species (Busack, 1986; García-París & Jockusch, 1999; Martínez-Solano, 2004; Real et al., 2005; Velo-Antón et al., 2008). Such taxonomic uncertainty mostly stems from the finding of discordant patterns of genetic variation between different molecular markers: mitochondrial DNA provides evidence of two highly divergent (pairwise genetic distances over 8%), geographically structured lineages (García-París & Jockusch, 1999; Martínez-Solano, 2004), whereas allozyme data found much lower genetic differentiation between them (Lanza et al., 1986; Zangari et al., 2006). Studies using sequences of nuclear markers did not find evidence of reciprocal monophyly between the two taxa,

Correspondence: I. Martínez-Solano (inigomsolano@gmail.com)

either (Velo-Antón et al., 2008; Pabijan et al., 2012). The cause of this discordance remains unclear and contact zones between both taxa have not been characterised in detail, so little is known about patterns of gene flow across taxa.

The use of hypervariable markers like microsatellites can provide relevant information on this issue by helping characterise the pattern, rate and geographic extent of admixture/hybridisation between the Iberian taxa. Microsatellites are DNA sequences composed of tandem repeats of 2–6 nucleotides in which alleles are defined by different number of repetitions. These highly variable markers have co-dominant inheritance and have become the markers of choice for population genetics studies applied to evolutionary, ecological and taxonomical questions as well as for conservation genetics. In this study, we report the isolation and characterisation of novel polymorphic microsatellite loci for the Iberian taxa *D. galganoi* and *D. jeanneae*. Cross-species amplification was assessed in all species of the genus *Discoglossus* and in the recently rediscovered and highly endangered relative *Latonia nigriventer* (Biton et al., 2013).

Sixteen specimens of *D. jeanneae* from the vicinity of its type locality, twenty-nine specimens from two populations of *D. galganoi* (one of them near its type locality) and forty-two specimens of three Iberian populations of *Discoglossus* of uncertain species assignment were collected for genotyping (for details see below). Genomic DNA was extracted from tail clips of larvae or toe clips of adults and juveniles with NucleoSpin Tissue-Kits (Macherey-Nagel). Additionally, specimens for all the extant Discoglossinae species were collected for cross-amplification tests. These included eight samples of *D. montalentii* from Corsica; seven samples of *D. sardus* from two localities in Corsica and three in Sardinia; twenty seven samples of *D. pictus* from three localities in Tunisia and one in NE Spain; eight samples of *D. scovazzi* from two localities in Morocco and one in

Table 1. Description of new microsatellite loci isolated in Iberian painted frogs (*Discoglossus*). Locus name, primer sequences (forward -top- and reverse -bottom-), fluorescent dye, repeat motif, multiplex reaction, number of alleles per locus (N_A) and size of amplified product (bp) are reported for each locus.

Locus	Primer sequence	Labelling dye	Repeat motif	Multiplex reaction	N_A	Size range (bp)
Dj3.5	5′ CAAGGAATGCTTTAGAGGCAG 3′ 5′ ACCCTCCGTTCTTTGCTAGG 3′	VIC	$(TTA)_8$	Multiplex 1	5	139–157
Dj3.23	5′ CCAGGTCAAGGGTGAGACAT 3′ 5′ TGTATTATTACCACATATGAGCCAC 3′	NED	$(AAT)_9$	Multiplex 1	10	111–141
Dj4.12	5′ CGAGTAAATTTCATAGCAATCCA 3′ 5′ CTGCTGACATCAAAAGTAGGTCA 3′	PET	$(ATCT)_5$	Multiplex 1	6	128–154
Dj3.6	5′ GGCCCTCCATTTGTGTAATG 3′ 5′ AAGGCAAGTATGGATGCAGT 3′	VIC	$(TAA)_6$	Multiplex 2	3	167–173
Dj4.27	5′ TCCCATGTCTAAATTGTGTAAATGA 3′ 5′ TTTCTTTTGTCCTGTTCTCTTTCTT 3′	NED	$(GATA)_{10}$	Multiplex 2	12	126–174
Dj4.4	5′ CACGCTCAGCTGCTGTTACT 3′ 5′ CAAATAATCAATGTTACCCCTAAAA 3′	PET	$(CTAT)_5$	Multiplex 2	6	120–160
Dj4.21	5′ TGTAGATGTCACAATACCCAAATG 3′ 5′ TGTAACATGCAGAATTCCCAA 3′	6-FAM	$(GATA)_{13}$	Multiplex 3	17	88–160
Dj4.3	5′ AGCTTCATTGTCCACAAGCC 3′ 5′ TGTGTAGGGAAAGACCGAATG 3′	VIC	$(TCTA)_8$	Multiplex 3	16	173–229
Dj4.6	5′ AGCCCTACCTCTGGATCATT 3′ 5′ CAGCAAGCTGTGCATCTCA 3′	NED	$(TAGA)_5$	Multiplex 3	15	141–205
Dj3.34	5′ CTCAATCTCCTGTTGGAGGC 3′ 5′ GGTCCAAGTTCCTGCTTGAC 3′	PET	$(TGG)_5$	Multiplex 3	3	160–175
Dj4.28	5′ AAAGTATTGGCATCGCCATC 3′ 5′ TAACAACCCCACCTGCCAT 3′	NED	$(CTAT)_{10}$	Multiplex 4	21	187–242
Dj4.23	5′ TCATATAGGAAAAGACTACCCCTGA 3′ 5′ AGTGGAACAGATCTGGTGAAGA 3′	PET	$(ATAG)_5$	Multiplex 4	5	110–124
DJ4.18	5′ CCCAGCAATTAGGCTCTGTT 3′ 5′ GCATTTCCAGTTTTCACAACG 3′	VIC	$(CAGA)_5$	Multiplex 4	2	119–123
Dj3.35	5′ AACAGACAGGATCAGGCAGG 3′ 5′ ACCCATGTATAGCGCTACGG 3′	NED	$(TAT)_7$	Multiplex 5	4	110–122
Dj3.2	5′ GCTGTGTATAGGTGCATGGG'3′ 5′ GCACTACGGAATATGTTGGCT 3′	PET	$(TGT)_9$	Multiplex 5	10	133–166
Dj3.24	5′AGGTCAGGTCTGGAAGCTCA 3′ 5′ CCATGCAAGGTGTGATTTTG 3′	VIC	$(GAG)_6$	Multiplex 6	2	147–150
Dj3.3	5′ GATGAGGTCATCGGCCTAAA 3′ 5′ TCCCTCTATTCTCTTTCTTTCTCC 3′	NED	$(ACA)_8$	Multiplex 6	8	166–193
Dj4.7	5′ TCACGTGAAGAAGGGAGGAC 3′ 5′ CTGTCCCTCTGAGCTCCATC 3′	PET	$(AGAT)_8$	Multiplex 6	15	106–220

Table 2. Cross-species amplification results in four *Discoglossus* species and in *Latonia nigriventer*: species name, number of samples genotyped per species (in parentheses) and number of alleles per locus.

Species (n)	Locus																	
	Dj3.5	Dj3.23	Dj4.12	Dj3.6	Dj4.27	Dj4.4	Dj4.21	Dj4.3	Dj4.6	Dj3.34	Dj4.28	Dj4.23	Dj4.18	Dj3.35	Dj3.2	Dj3.24	Dj3.3	Dj4.7
D. montalentii (8)	-	-	5	1	-	2	-	-	-	-	5	1	2	2	-	1	-	-
D. sardus (7)	-	-	-	1	-	3	5	-	-	-	-	-	-	3	1	1	-	-
D. pictus (27)	-	7	-	3	-	13	-	-	-	-	6	-	-	7	4	1	-	-
D. scovazzi (8)	3	2	5	1	8	-	1	-	-	2	2	-	9	-	4	1	-	-
L. nigriventer (3)	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-

Spain (Ceuta); and three samples of *L. nigriventer* from Israel.

The microsatellite library was developed from tissue from one tadpole (voucher: IMS1176), collected in the vicinity of the locality of Alcalá de los Gazules, Cádiz, Spain, near the type locality of *D. jeanneae*. It was constructed at the Sequencing Genotyping Facility, Cornell Life Sciences Core Laboratory Center (CLC). Genomic DNA was extracted with Qiagen DNeasy Blood and Tissue Kits and digested with the restriction enzyme Hinc II. Subsequently, the digested DNA was ligated to linkers and later enriched for microsatellites by hybridisation with 3' biotinylated oligonucleotide repeat probes. The enriched fragments were captured by streptavidin-coated magnetic beads and amplified by PCR. Then, amplified PCR products were ligated to Titanium 454 Rapid Library MID adapter and sequenced by Titanium 454 sequencing at CLC. Titanium adapters were trimmed of MID-sorted 454 reads. The reads obtained were assembled with SeqMan Pro (Lasergene v.8.1.1, DNASTAR, Inc.), generating a fasta (.fas) file of 30,748 assembled contigs.

Sequences containing microsatellites were scanned with iQDD v.1.3 (Meglécz et al., 2010). This program implements Clustalw2 (Larkin et al., 2007), BLAST (ftp://ftp.ncbi.nih.gov/blast/executables/) and Primer3 (Rozen & Skaletsky, 2000) using ActivePerl (http://www.activestate.com/activeperl/). First, sequences longer than 80 bp containing tri- and tetra- like microsatellite motifs and including a minimum of five repetitions were selected. Secondly, a sequence similarity detection procedure was carried out to remove redundancy and eliminate problematic sequences for microsatellite amplification. This step was performed through an 'All against all' BLAST analysis (Altschul et al., 1997; e-value 1E-40) with default settings. Finally, 63 primer pairs flanking regions with microsatellite motifs were designed with a minimum length of flanking region of 20 bp and a range size of PCR products between 90–320 bp, with an optimal melting temperature of 60°C to facilitate multiplexing.

Polymerase chain reaction (PCR) was used to perform an initial test of the 63 pairs of primers in eight samples of *D. jeanneae* and *D. galganoi*. All PCRs were performed in a total volume of 15 µl, including 25 ng of template DNA, 5x GoTaq Flexi buffer (PROMEGA), 1.5 mM $MgCl_2$, 0.3 mM dNTP, 0.3 µM of each primer and 0.5 U GoTaq Flexi DNA polymerase (PROMEGA). PCR cycling conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at a 60°C for 45 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. PCR products were visualised on 2.5 % agarose gels.

Consistent amplification across individuals was obtained in a total of 24 primers pairs, which were selected for subsequent screening. These primers were combined in multiplex reactions using Multiplex Manager v.1.2 (Holleley & Geerts, 2009) and forward primers were 5'-labelled with the fluorescent dyes 6-FAM, PET, NED and VIC (Table 1). Six multiplex reactions were performed using Type-it Microsatellite PCR kits (Qiagen). All reactions

Table 3. Populations of *D. jeanneae* and *D. galganoi* screened for variation with the microsatellites developed in the present study, including locality information, population code, number of samples per population (*n*), geographic coordinates (latitude and longitude), and estimates of genetic diversity (number of alleles, observed and expected heterozygosity).

Species	Locality	Code	*n*	Latitude	Longitude	N_A	H_O	H_E
Discoglossus sp.	Valdelaguna, Madrid, Spain	VAL	14	40°11'N	3°22'W	2.625	0.286	0.298
Discoglossus sp.	Villamanta, Madrid, Spain	VIL	13	40°18'N	4°07'W	4.063	0.466	0.485
Discoglossus sp.	Villaviciosa de Odón, Madrid, Spain	SAC	15	40°21'N	3°57'W	4.250	0.509	0.527
D. jeanneae	Alcalá de los Gazules, Cádiz, Spain	ALC	15	36°23'N	5°39'W	4.813	0.551	0.570
D. galganoi	Valverde, Fundão, Castelo Branco, Portugal	VLV	16	40°09'N	7°27'W	4.750	0.537	0.554
D. galganoi	Silves, Faro, Portugal	SIL	13	37°15'N	8°21'W	3.500	0.431	0.449

were performed in a total volume of 15 µl, containing 7.5 µl of the Master Mix, 1.2 µl of primer mix (0.2 µM of each primer), and 5.3 µl of RNase-free H_2O. The PCR cycling profile consisted of an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 90 seconds, and extension at 72°C for 30 seconds, with a final extension at 60°C for 10 minutes. Genotyping was carried out on an ABI PRISM 3730 sequencer with the GeneScan 500 LIZ size standard (Applied Biosystems), and alleles were scored manually using GeneMapper v.4.0 (Applied Biosystems). Among loci that consistently amplified in the Iberian populations (PCR success rate >99%), those that were polymorphic were tested for cross-amplification in the other four species of *Discoglossus* and in *Latonia nigriventer* (Table 2).

Number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities were estimated for each locus and population with GenAlEx 6.5b5 (Peakall & Smouse, 2006). Microchecker v.2.2.3 (van Oosterhout et al., 2004) was used to test for the presence of stuttering, large allele dropout and null alleles in each population and locus, with a 99% confidence interval and 1,000 randomisations. Deviations from Hardy-Weinberg equilibrium (HWE) and evidence of linkage disequilibrium (LD) were tested between all pairs of loci using Genepop on the web (http://genepop.curtin.edu.au/; Raymond & Rousset, 1995; Rousset, 2008). Significance values for all multiple tests were evaluated applying a sequential Bonferroni correction (Rice, 1989).

Eighteen of the 24 microsatellite loci tested were polymorphic in six Iberian populations of *Discoglossus* (*D. jeanneae*/*D. galganoi*; Table 1, GenBank Accession numbers: KJ546860–KJ546877). Null alleles were detected in loci Dj4.3 and Dj3.3 in VIL and SAC, Dj4.3, Dj3.3 and Dj4.7 in ALC and in loci Dj3.34 and Dj3.3 in VLV. The observed number of alleles ranged from 2 to 21 per locus, and sizes ranged from 88 to 242 bp. Locus Dj4.12 only amplified in the populations of *D. galganoi* VAL and SIL. The average observed and expected heterozygosities were similar between the six populations (VAL: 0.286/0.298; VIL: 0.466/0.485; SAC: 0.509/0.527; ALC: 0.551/0.570; VLV: 0.537/0.554; SIL: 0.431/0.449) (Table 3). Deviations from HWE were detected in loci Dj4.3, Dj3.3 in VIL, SAC and ALC, and Dj3.34 and Dj3.3 in VLV. We found no evidence of linkage disequilibrium in any loci combination in either population.

Among the eighteen loci tested for cross-amplification in the other four species of *Discoglossus* and in *L. nigriventer*, four didn't amplify in any of them. Amplification in *D. scovazzi* was the most successful, with 11 loci, 8 of which were polymorphic. The results were similar between *D. pictus* and *D. montalentii,* with seven and eight amplifying loci, of which six and five were polymorphic, respectively (see Table 2). In the case of *D. sardus,* only three markers were polymorphic and an additional three amplified but were monomorphic. Finally, these markers were tested in the recently rediscovered *L. nigriventer*. Only two microsatellite loci amplified, and both of them were monomorphic in the three samples genotyped.

These new markers will be useful in addressing a variety of questions related to species delineation, evolutionary history, population structure and conservation of Iberian *Discoglossus.* More detailed studies describing patterns of genetic diversity and structure across their ranges are underway, with a special interest in comparison with published data on mtDNA variation. These new loci can also be applied to address similar questions in other species in the genus, especially in the North African species *D. scovazzi* and *D. pictus*. The latter is an invasive species rapidly expanding its range in SE France and NE Spain, where negative impacts on native amphibian communities have been reported (Richter-Boix et al., 2013). The high level of cross-amplification success in these species is in accordance with their closer phylogenetic affinity to Iberian taxa (Pabijan et al., 2012; Biton et al., 2013). On the other hand, as expected, success was much lower in more distant taxa, like *D. sardus* and *D. montalentii*, and especially so in *Latonia.* This species is listed as Critically Endangered by the IUCN, since its whole known range includes just about 2 km^2 in the Hula Valley in Israel and the total known population is in the order of a dozen individuals (Biton et al., 2013; IUCN, 2013). Genetic diversity is presumed to be extremely low in this species, making it challenging to find highly polymorphic molecular markers that may aid in their conservation.

Acknowledgements: We thank J. Barbadillo, J. Ben Hassine, D. Buckley, H. Clavero, K. Ghali, C. Grande, M. Lapeña, J.A. Lucas, G. Llorente, R. Pereira, E. Pujol, E. Recuero, R. Sermier and S. Nouira for help collecting samples and S. Bogdanowicz at Cornell University for help with the microsatellite library. This research was funded by grants CGL2008-04271-C02-01/BOS and CGL2011-28300 (Ministerio de Ciencia e Innovación, Ministerio de Economía y Competitividad, Spain, and FEDER) and PPII10-0097- 4200 (Junta de Comunidades de Castilla la Mancha and FEDER) to IMS. JGR is supported by the Consejo Superior de Investigaciones Científicas of Spain (CSIC) and the European Social Fund (ESF) (JAE-pre PhD fellowship); IMS is funded by Project "Biodiversity, Ecology and Global Change" co-financed by North Portugal Regional Operational Programme 2007/2013 (ON.2-O Novo Norte), under the National Strategic Reference Framework (NSRF), through the European Regional Development Fund (ERDF) and DS is supported by a post-doctoral grant (SFRH/BPD/66592/2009) funded by Fundação para a Ciência e Tecnologia (FCT, Portugal) under the Programa Operacional Potencial Humano - Quadro de Referência Estratégico Nacional funds from the European Social Fund and Portuguese Ministério da Educação e Ciência.

REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.

Biton, R., Geffen, E., Vences, M., Cohen, O., et al. (2013). The

rediscovered Hula painted frog is a living fossil. *Nature communications* 4, 1959.

Busack, S.D. (1986). Biochemical and morphological differentiation in Spanish and Moroccan populations of *Discoglossus* and the description of a new species from southern Spain. *Annals of Carnegie Museum of Natural History* 55, 41–61.

Capula, M. & Corti, C. (1993). Morphometric variation and divergence in the West Mediterranean *Discoglossus* (Amphibia, Discoglossidae). *Journal of Zoology* 231, 141–156.

Fromhage, L., Vences, M. & Veith, M. (2004). Testing alternative vicariance scenarios in Western Mediterranean discoglossid frogs. *Molecular Phylogenetics and Evolution* 31, 308–322.

García-París, M. & Jockusch, E.L. (1999). A mitochondrial DNA perspective on the evolution of Iberian *Discoglossus* (Amphibia: Anura). *Journal of Zoology* 248, 209–218.

Holleley, C.E. & Geerts, P.G. (2009). Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. *BioTechniques* 46, 511–517.

IUCN SSC Amphibian Specialist Group. (2013). *Discoglossus nigriventer*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1. Available from: <http://www.iucnredlist.org>. Accessed 1 October 2013.

Lanza, B., Nascetti, G., Capula, M. & Bullini, L. (1986). Les discoglosses de la region mediterraneenne occidentale. *Bulletin de la Société Herpétologique de France* 40, 16–27.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., et al. (2007). ClustalW and ClustalX version 2.0. *Bioinformatics* 23, 2947–2948.

Martínez-Solano, I. (2004). Phylogeography of Iberian *Discoglossus* (Anura, Discoglossidae). *Journal of Zoological Systematics and Evolutionary Research* 42, 298–305.

Meglécz, E., Costedoat, C., Dubut, V., Gilles, A., et al. (2010). QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* 26, 403–404.

Pabijan, M., Crottini, A., Reckwell, D., Irisarri, I., et al. (2012). A multigene species tree for Western Mediterranean painted frogs (*Discoglossus*). *Molecular Phylogenetics and Evolution* 64, 690–696.

Peakall, R.O.D. & Smouse, P.E. (2006). Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6, 288–295.

Raymond, M. & Rousset, F. (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86, 248–249.

Real, R., Barbosa, A.M., Martínez-Solano, I. & García-París, M. (2005). Distinguishing the distributions of two cryptic frogs (Anura: Discoglossidae) using molecular data and environmental modeling. *Canadian Journal of Zoology* 83, 536–545.

Rice, W.R. (1989). Analyzing tables of statistical tests. *Evolution* 43, 223–225.

Richter-Boix, A., Garriga, N., Montori, A., Franch, M., San Sebastián, O., et al. (2013). Effects of the non-native amphibian species *Discoglossus pictus* on the recipient amphibian community: niche overlap, competition and community organization. *Biological Invasions* 15, 799–815.

Rousset, F. (2008). Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. *Molecular Ecology Resources* 8, 103–106.

Rozen, S. & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics methods and protocols*, 365–386. Krawetz, S. & Misener, S. (eds.). New Jersey: Totowa Humana Press.

van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. & Shipley, P. (2004). Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4, 535–538.

Velo-Antón, G., Martínez-Solano, I. & García-París, M. (2008). ß-fibrinogen intron 7 variation in *Discoglossus* (Anura: Discoglossidae): implications for the taxonomic assessment of morphologically cryptic species. *Amphibia-Reptilia* 29, 523–533.

Vences, M. & Glaw, F. (1996). Further investigations on *Discoglossus* bioacoustics: Relationships between *D. galganoi galganoi*, *D. g. jeanneae* and *D. pictus scovazzi*. *Amphibia-Reptilia* 17, 333–340.

Zangari, F., Cimmaruta, R. & Nascetti, G. (2006). Genetic relationships of the Western Mediterranean painted frogs based on allozymes and mitochondrial markers: evolutionary and taxonomic inferences (Amphibia, Anura, Discoglossidae). *Biological Journal of the Linnean Society* 87, 515–536.

Accepted: 20 March 2014