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Species assignment in the *Pelophylax ridibundus* x *P. perezi* hybridogenetic complex based on 16 newly characterised microsatellite markers

Gregorio Sánchez-Montes[1,2], Ernesto Recuero[3], Jorge Gutiérrez-Rodríguez[2], Ivan Gomez-Mestre[4] & Iñigo Martínez-Solano [4,5,6]

[1]*Departamento de Biología Ambiental, Facultad de Ciencias, Universidad de Navarra, c/ Irunlarrea, 1, 31008 Pamplona, Spain*

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

[3]*Departamento de Ecología de la Biodiversidad, Instituto de Ecología, Universidad Nacional Autónoma de México, Ap. Postal 70-275, Ciudad Universitaria, México DF, 04510, Mexico*

[4]*Ecology, Evolution, and Development Group, Department of Wetland Ecology, Doñana Biological Station, CSIC, c/ Américo Vespucio, s/n, 41092 Seville, Spain*

[5]*Instituto de Investigación en Recursos Cinegéticos (CSIC-UCLM-JCCM), Ronda de Toledo, s/n, 13005 Ciudad Real, Spain*

[6]*CIBIO-InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, Universidade do Porto, 4485-661 Vairão, Portugal*

Pelophylax perezi is an Iberian green waterfrog with high tolerance to habitat alteration that at times shows local population growth and demographic expansion, even where other species decline. However, pond destruction, invasive predators and hybridisation with other European waterfrog species (*P. ridibundus*) threaten many of its populations across its range. Hybrids of *P. perezi* and *P. ridibundus* (*P.* kl. *grafi*) can breed successfully with the former parental species after discarding the whole *P. perezi* genome in the germinal line, thus representing a sexual parasite for *P. perezi*. However, little is known about the extent of the contact zone of this hybridogenetic complex. Due to the morphological similarity of the three taxa, molecular tools are needed to delineate their respective ranges. Here we characterise a set of 16 microsatellite markers specifically developed for *P. perezi*. These markers showed moderate to high polymorphism (2–17 alleles/locus) in two populations from central Spain (*n*=20 and *n*=23), allowing individual identification of frogs. Seven of these markers cross-amplified in individuals of *P. ridibundus* from southern France (3–8 alleles/locus). These markers were used to genotype samples along a transect from southern France to eastern Spain, encompassing both pure and hybrid individuals. Sample assignment to each taxon was based on the new microsatellite loci and compared with nuclear and mitochondrial sequence data. Our results show that these markers are useful to distinguish *P. ridibundus*, *P. perezi* and the hybrid form *P.* kl. *grafi* from each other, even when sample sizes are low. The newly characterised markers will also be useful in demographic and phylogeographic studies in *P. perezi* and are thus a valuable tool for evolutionary and conservation oriented research.

Key words: cross-amplification, hybridisation, microsatellites, *Pelophylax* kl. *grafi*, *Pelophylax perezi*, *Pelophylax ridibundus*

INTRODUCTION

Perez's Frog, *Pelophylax perezi* (López-Seoane, 1885), is a medium sized green waterfrog endemic to the Iberian Peninsula and southern France. It has been introduced into the Balearic, Canary and Azores Archipelagos and in two localities in the UK (Bosch et al., 2009). *Pelophylax perezi* shows great adaptability to breed in almost every kind of water body and exhibits tolerance to a wide range of ecological and physicochemical conditions. Moreover, its larvae respond to predators altering their behaviour, shape and degree of pigmentation hence improving survival (Gomez-Mestre & Díaz-Paniagua, 2011). This plasticity and degree of tolerance to environmental degradation may explain the success of this species in humanised areas. It is remarkable that Perez's frog persists or even thrives in the same locations where other amphibian species show dramatic negative trends (Martinez-Solano et al., 2003a, b). However, despite this resilience, *P. perezi* is locally also at risk from invasive predators due to lack of innate recognition and habitat overlap (Cruz & Rebelo, 2005; Gomez-Mestre & Díaz-Paniagua, 2011), habitat destruction and hybridisation with other species. Demographic studies are essential to identify populations at risk of loss of genetic diversity for conservation purposes. Relevant parameters such as breeding success, effective population size and gene flow/admixture can be estimated with the help of molecular markers. Among these, microsatellites are especially useful in local-scale studies because of their high polymorphism (Hotz et al., 2001).

As in other *Pelophylax* species, *P. perezi* is susceptible

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

to hybridisation and introgression with other green frog species (Graf et al., 1977; Uzzell & Tunner, 1983). In fact, some areas in southern France and north-eastern Spain harbour viable *P. ridibundus* x *P. perezi* hybrids (F_1 hemiclones formed through hybridogenesis and named as *Pelophylax* klepton *grafi*) that are found along with one or both parental species in those locations, thus forming an hybridogenetic complex (Dubois & Ohler, 1994; Arano et al., 1995; Crochet et al., 1995). *Pelophylax* kl. *grafi* is considered a synonym of *P. perezi* by some authors (Frost, 2014), but it discards the whole *P. perezi* genome in its germinal line and is able to maintain a hybrid lineage by backcrossing with *P. perezi* individuals. It thus represents a sexual parasite, capable of reducing genetic diversity in populations of *P. perezi*. It originated either from hybridisation of *P. perezi* with ancestral, isolated *P. ridibundus* populations, or from hybridisation with *P*. kl. *esculentus*, another klepton involving *P. ridibundus* and *P. lessonae* (Crochet et al., 1995). *Pelophylax* kl. *grafi* is currently listed as "Near Threatened" in IUCN's Red List of Threatened Species due to "an observed decline as a result of competition from the introduced species *P. ridibundus"* (Tejedo et al., 2009), i.e. by the introduction of *P. ridibundus* from central and eastern Europe and Asia. However, the precise distribution limits of the hybrids and their parental species in their contact zone are mostly unknown because species identification in the field, based on morphological characters, is problematic (see for instance, Petitot et al., 2014). Hybridogenesis typically implies asymmetrical participation of the sexes from the different species of the complex, but its biological mechanisms, the relative performance of individuals of the three taxa in different conditions, and the ecological and evolutionary consequences remain largely unexplored (Berger et al., 1988; Hotz et al., 1992). Molecular tools may thus be a valuable conservation tool for delineating parental populations and hybrid zones and for tracing the history of pure and hybrid lineages.

In this paper, we describe a set of 16 microsatellite loci specifically developed and optimised for *P. perezi* and evaluate their utility for population studies as general indicators of genetic diversity. We then assess cross-species amplification success in a population of *P. ridibundus* from southern France as well as in additional putative samples of *P. perezi* from the eastern part of the Iberian range, including the area north of the Ebro River, where hybridisation events between the two species have been previously detected (Arano et al., 1995). With help

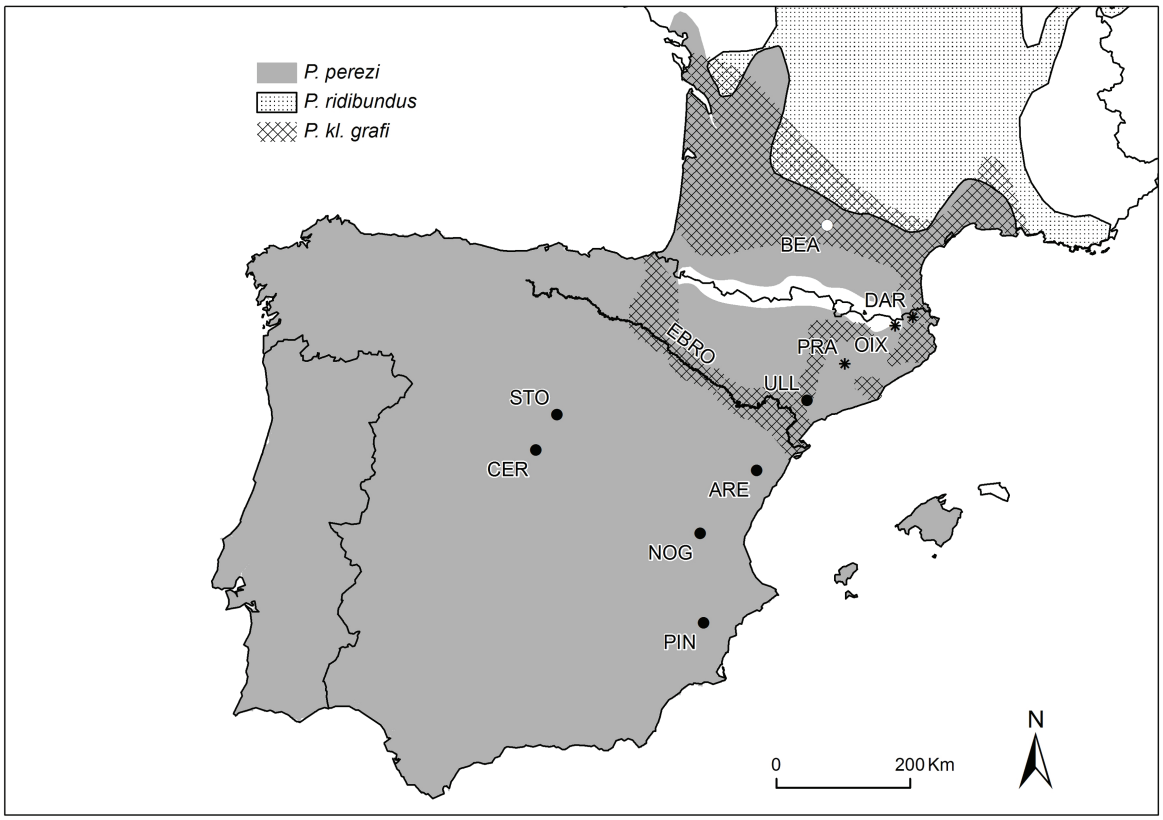


Fig. 1. Map showing the approximate ranges of *P. ridibundus* (dotted area), *P. perezi* (grey), and *P.* kl. *grafi* (mesh), indicating the location of sampled populations. Ranges of the two parental species are based on IUCN assessments (Bosch et al., 2009; Kuzmin et al., 2009), whereas for *P.* kl. *grafi* we incorporated information from Rivera et al. (2011). The course of the Ebro River, the major corridor for dispersal of *P.* kl. *grafi* in Iberia, is also indicated. The contact between eastern and western Iberian *grafi* nuclei through the Ebro River is assumed, but it is not fully documented. Sampling localities are represented with different symbols based on taxonomic assignment of individuals analysed: *P. perezi* (dark circles), *P. ridibundus* (white circle), and the hybridogenetic complex (dark asterisks). STO = Santo Tomé, CER = Cerceda, BEA = Beauzelle, DAR = Darnius, OIX = Oix, PRA = Prades, ULL = Ulldemolins, ARE = Ares del Maestre, NOG = Las Nogueras, PIN = Pinoso.

Table 1. Characterisation of 16 microsatellite loci in *Pelophylax perezi,* including primer sequences, labelling dye, repeated motif, multiplex reaction and size range. Annealing temperature was 60°C in all cases. *n*=sample size, Na=allelic richness, H_O *and* H_E=observed and expected heterozygosities in the populations of Santo Tomé / Cerceda, respectively. Cross-amplification in *P. ridibundus* (Pr) is indicated with the sign "+". GB: GenBank accession numbers.

Locus	Primer sequence	Labelling dye	Repeated motif	Multiplex reaction	Size range (bp)	n	Na	H_O	H_E	*Pr*	GB
Pper4.25	5′ TCCCTTCTAGTGCTGTAACTTCG 3′ 5′ AGTTCATCTGCAGTTCCTACATG 3′	6-FAM	$(AGAT)_8$	1	199-385	20/23	11/17	0.8/0.87	0.84/0.92	-	KT166015
Pper4.15	5′ ACATATTGTGCTGCTCCATCAAG 3′ 5′ AATTTCTTCAGTGCTGTCATGTC 3′	VIC	$(AGAT)_8$	1	177-236	20/23	8/11	0.8/0.96	0.84/0.86	-	KT166016
Pper4.28	5′ CATGTACAGCTGACTTTAGAGCC 3′ 5′ TTCTTTCCAATTTGAGACTCGGG 3′	NED	$(AAGG)_5$	1	201-251	20/23	2/5	0.3/0.61	0.48/0.62	-	KT166017
Pper3.9	5′ CAACATATCTTCCCGAATGAGGC 3′ 5′ GTTTCTCTCAGTCTAGTTGGTGC 3′	PET	$(AAG)_6$	1	201-256	20/23	6/7	0.6/0.87	0.56/0.76	-	KT166018
Pper4.5	5′ TGTGCGCTATCCTCTGTAGTTAG 3′ 5′ TGAATCCTGGCATTGTCATCTTG 3′	VIC	$(AAAC)_6$	2	147-160	20/23	4/3	0.55/0.7	0.66/0.62	+	KT166019
Pper4.16	5′ AGAGCAGATATACCACACTCCAG 3′ 5′ ACCTCAAGCATTTATAGACCAGC 3′	NED	$(AGAT)_9$	2	139-184	20/23	5/10	0.7/0.83	0.74/0.85	-	KT166020
Pper3.24	5′ ATGTGGAGACTATCAGCAGACAG 3′ 5′ CAAGTCTTGACTGTTCATACCGG 3′	PET	$(AAC)_7$	2	251-274	20/23	6/7	0.5/0.83	0.61/0.78	+	KT166021
Pper4.20	5′ TCTTAGCAGTGACAGATGTGAAC 3′ 5′ TCTTAGTGCAGATTAGGGACCTG 3′	VIC	$(AAGT)_6$	3	220-224	19/23	1/2	0/0.43	0/0.49	-	KT166022
Pper3.22	5′ ACTGTCATCTGGTCTGGTATCAC 3′ 5′ ACACTAATTGTCCTCCTGTAGAAC 3′	NED	$(ACT)_9$	3	359-379	19/23	5/5	0.53/0.61	0.54/0.49	+	KT166023
Pper4.13	5′ AGAGACCATATATCGGAGCCATC 3′ 5′ TGGCAAATCACTCCACTTAACAG 3′	PET	$(AGAT)_{10}$	3	442-494	19/23	5/11	0.79/0.78	0.74/0.87	-	KT166024
Pper4.7	5′ TACCTCTTCTGCTGATCTCTTGG 3′ 5′ AAGCAATTTATCAAGCAGGAGGG 3′	NED	$(AGAT)_9$	4	292-346	20/22	6/15	0.8/1	0.79/0.89	+	KT166025
Pper3.1	5′ TTGCCAGCAGAAGAGAACATTAC 3′ 5′ TCTCACAGACATCGCATTTGATC 3′	PET	$(AGG)_9$	4	340-364	20/23	5/6	0.95/0.61	0.69/0.67	-	KT166026
Pper4.23	5′ AGCTGTCAAAGGATGTCATGTTC 3′ 5′ TCAGGTGAGAGATCGAAATACCC 3′	6-FAM	$(AGAT)_9$	5	440-492	20/23	7/12	0.65/0.7	0.73/0.88	+	KT166027
Pper4.29	5′ CTGTGCTACGAGGATTGTAATGG 3′ 5′ TTCATTCTCTGTGTCGTGAATGC 3′	VIC	$(AAAG)_7$	5	321-349	20/23	5/8	0.55/0.91	0.51/0.80	+	KT166028
Pper3.23	5′ ACTTGTATCATCTTTCTCTGCGC 3′ 5′ TTTCTGCCCAATTCTACTACTGC 3′	NED	$(ACT)_6$	5	154-181	20/23	3/4	0.45/0.78	0.60/0.70	-	KT166029
Pper4.24	5′ TTTCCCTATTGCCTATGAACTGC 3′ 5′ AGTGCTATGGTTGGGATTTGAAC 3′	PET	$(AGAT)_{10}$	5	203-262	20/23	7/9	0.85/0.91	0.80/0.84	+	KT166030

from additional molecular markers (sequences of the mitochondrial gene cytochrome oxidase I and the nuclear gene tyrosinase), we discuss the utility of the newly developed microsatellites in identifying hybrids (*P.* kl. *grafi*) and both parental species of the complex (*P. perezi* and *P. ridibundus*).

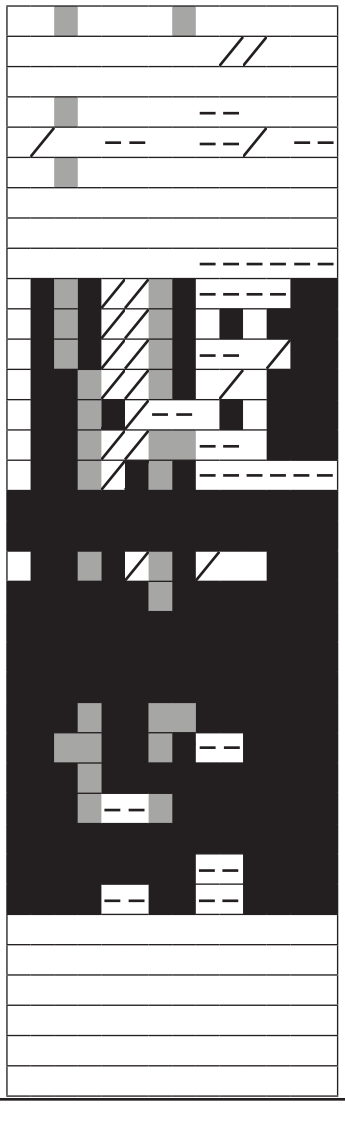
MATERIALS AND METHODS

An enriched partial genomic library was generated from DNA of a single tadpole of *P. perezi* collected in Valdemanco, central Spain (40°51' N, 3°38' W). The library was constructed at the Sequencing Genotyping Facility, Cornell Life Sciences Core Laboratory Center (CLC) following the method described in Gutiérrez-Rodríguez & Martínez-Solano (2014). A total of 60 loci containing microsatellite motifs (30 trimers and 30 tetramers) between 4 and 10 repetitions long were selected for further screening.

Genomic DNA was extracted from tail tips of larvae and toe tips of newly metamorphosed froglets and adult frogs with NucleoSpin Tissue-Kits (Macherey-Nagel). PCR reactions were performed in a total volume of 15 µl, including approximately 25 ng of template DNA, 5x GoTaq Flexi buffer (PROMEGA), 3.33 mM $MgCl_2$, 0.33 mM dNTP, 0.33 µM of each primer and 0.5U GoTaq Flexi DNA polymerase (PROMEGA). PCR cycling consisted of initial denaturation (95°C, 5 min), 40 cycles of denaturation (95°C, 45 s), annealing (60°C, 45 s), and extension (72°C, 45 s), with a final extension step (72°C, 10 min). PCR products were visualised on 2% agarose gels.

Of the 60 pairs of primers tested, 20 amplified consistently showing unambiguous bands and were chosen for subsequent multiplex reactions. Forward primers were labelled with fluorescent dyes (6-FAM, VIC, NED and PET) for use in five multiplex reactions designed with Multiplex Manager v.1.2 (Holleley & Geerts, 2009)

Table 2. Results of individual assignment analyses by means of mitochondrial (*cox1*), nuclear (*TYR*) and seven microsatellite loci (prob.: assignment probabilities in NewHybrids analyses). The 14 alleles of each microsatellite genotype are coded as private *P. perezi* allele (black), private *P. ridibundus* allele (white), shared by *P. perezi* and *P. ridibundus* (grey), exclusive of mixed individuals (diagonal) or missing data (horizontal). (*): for these two samples, mtDNA-based assignment is based on sequences from a different marker (ND2, unpublished data).

Sample	Population	mtDNA	TYR	Microsatellite (prob.)	Diagnostic alleles
Rz179	Beauzelle	*P. ridibundus*	*P. ridibundus*	*P. ridibundus* (>0.99)	
Rz181	Beauzelle	*P. perezi*	*P. ridibundus*	*P. ridibundus* (>0.99)	
Rz182	Beauzelle	*P. ridibundus*	*P. ridibundus*	*P. ridibundus* (>0.99)	
Rz183	Beauzelle	*P. ridibundus*	*P. ridibundus*	*P. ridibundus* (>0.99)	
Rz184	Beauzelle	*P. perezi*	*P. ridibundus*	*P. ridibundus* (>0.99)	
Rz185	Beauzelle	*P. ridibundus*	*P. ridibundus*	*P. ridibundus* (>0.99)	
Rz186	Beauzelle	*P. ridibundus*	*P. ridibundus*	*P. ridibundus* (>0.99)	
Rz187	Beauzelle	*P. ridibundus*	*P. ridibundus*	*P. ridibundus* (>0.99)	
Rz188	Beauzelle	*P. ridibundus*	---	*P. ridibundus* (>0.99)	
Rz143	Oix	*P. perezi*	---	*P. ridibundus* (0.97)	
Rz144	Oix	*P. perezi*	*P. ridibundus*	*P. ridibundus* (0.83)	
Rz145	Oix	*P. perezi*	*P. ridibundus*	*P. ridibundus* (0.98)	
Rz161	Darnius	*P. perezi*	---	*P.* kl. *grafi* (0.86)	
Rz162	Darnius	*P. perezi*	*P. ridibundus*	*P.* kl. *grafi* (0.93)	
Rz304	Prades	*P. perezi*	*P. ridibundus*	*P. ridibundus* (0.64)	
Rz305	Prades	*P. perezi*	*P. ridibundus*	*P.* kl. *grafi* (0.99)	
Rz306	Prades	*P. perezi*	*P. perezi*	*P. perezi* (>0.99)	
Rz307	Prades	*P. perezi* *	*P. perezi*	*P. perezi* (>0.99)	
Rz308	Prades	*P. perezi* *	*P. ridibundus*	*P.* kl. *grafi* (0.98)	
Rz193	Ulldemolins	*P. perezi*	*P. perezi*	*P. perezi* (>0.99)	
Rz194	Ulldemolins	*P. perezi*	---	*P. perezi* (>0.99)	
Rz295	Ares del Maestre	*P. perezi*	*P. perezi*	*P. perezi* (>0.99)	
Rz296	Ares del Maestre	*P. perezi*	*P. perezi*	*P. perezi* (>0.99)	
Rz297	Ares del Maestre	*P. perezi*	*P. perezi*	*P. perezi* (>0.99)	
Rz279	Las Nogueras	*P. perezi*	*P. perezi*	*P. perezi* (>0.99)	
Rz280	Las Nogueras	*P. perezi*	*P. perezi*	*P. perezi* (>0.99)	
Rz281	Las Nogueras	*P. perezi*	*P. perezi*	*P. perezi* (>0.99)	
Rz273	Pinoso	*P. perezi*	*P. perezi*	*P. perezi* (>0.99)	
Rz275	Pinoso	*P. perezi*	*P. perezi*	*P. perezi* (>0.99)	
Rz276	Pinoso	*P. perezi*	*P. perezi*	*P. perezi* (>0.99)	
Rz18	Madrid	---	*P. perezi*	---	
Rz163	Madrid	*P. perezi*	---	---	
Rz166	A Coruña	*P. perezi*	*P. perezi*	---	
LAR8	Morocco	*P. saharicus*	*P. saharicus*	---	
24TU	Turkey	*P. ridibundus*	*P. ridibundus*	---	
BOS19.1	Bosnia	*P. ridibundus*	*P. ridibundus*	---	

(see Table 1). PCR reactions were performed using Type-it Microsatellite PCR kits (Qiagen). All reactions were run in a total volume of 15 µl, containing 7.5 µl of Master Mix, 1.2 µl of each primer mix (0.16 µM of each primer, except primers for *Pper4.7*, *Pper3.1* and *Pper4.23*, which were added in double concentration, 0.32 µM), and 5.3 µl of RNase-free H_2O. The PCR cycling conditions were: 95°C for 5 min, 30 cycles at 95°C for 30 s, 60°C for 90 s, and 72°C for 30 s, with a final extension at 60°C for 30 min. Genotyping was performed on an ABI PRISM 3730 sequencer with the GeneScan 500 LIZ size standard (Applied Biosystems). Allele peaks were assigned manually in GeneMapper v.4.0 (Applied Biosystems). Four of the 20 loci did not show assignable peaks and were thus discarded.

These new 16 molecular markers (GenBank accession numbers in Table 1) were tested in 43 individuals from two Iberian populations of *P. perezi* in Central Spain (Cerceda, Madrid, 40° 43' N, 3° 57' W, *n*=23, and Santo Tomé del Puerto, Segovia, 41° 12' N, 3° 35' W, *n*=20) (Fig. 1). Additionally, samples were collected along a north-south transect from southern France, through Catalonia and Comunidad Valenciana in eastern Spain, in order to capture pure parentals of *P. ridibundus* and *P. perezi* and their hybrids, *P.* kl. *grafi* (see Fig. 1 and Table 2). Since most samples were collected from metamorphs or larvae and thus morphological characters could not be used to unambiguously diagnose species, species assignment was aided by genotyping with mtDNA (*cox1*) and one nuclear marker, tyrosinase (*TYR*). These markers were amplified using primers and protocols described in Recuero et al. (2007) and Bossuyt & Milinkovitch (2000). For reference, we used samples of *P. ridibundus* from Bosnia and Turkey, one sample of *P. saharicus* (a close relative of *P. perezi*, see for instance Uzzell & Tunner, 1983; Akın et al., 2010) from Morocco, and two additional samples of *P. perezi* from Galicia (near the type locality of the species), and Madrid, in central Spain (in this case, samples from two different localities in Madrid were sequenced, one for each marker, see sample codes in Table 2). Sequences were edited with Sequencher v.5.0 (Gene Codes Corp., USA) and aligned by hand. Gene trees for each marker were inferred with the software BEAST v.1.8.1 (Drummond et al., 2012). Optimal partitioning strategies for each marker and associated models of nucleotide substitution were simultaneously selected with the software PartitionFinder v.1.1.1 (Lanfear et al., 2012). Three partitions were specified for *cox1*, corresponding to first (HKY+G), second (TrNef) and third (HKY) codon positions; and two partitions were defined in *TYR* sequences, corresponding to first plus third positions (K80+I), and second positions (HKY+G). Analyses in BEAST were run specifying a Yule coalescent prior and assuming a strict molecular clock. Parameter estimates were inspected to check for convergence and adequate Effective Sample Sizes (ESSs) in Tracer v.1.6 (Rambaut et al., 2014); subsequently, after removing 10% of the resulting trees as burn-in, the remaining trees were summarised with TreeAnnotator v.1.8.1 (distributed as part of the BEAST package). All new sequences were deposited in GenBank under accession numbers KT879303-KT879366.

Micro-Checker v.2.2.3 (van Oosterhout et al., 2004) was used to test for evidence of stuttering, large allele dropout and presence of null alleles in each population with sample size >5. Number of alleles (Na), observed (H_O) and expected (H_E) heterozygosity were calculated for each locus and population using GenAlEx 6.5 (Peakall & Smouse, 2012). We also used GenAlEx to estimate the power of resolution for individual identification of this set of microsatellite loci in the populations of Santo Tomé and Cerceda by calculating the Probability of Identity (PI) and another, more conservative estimate that accounts for possible relatives included in the sample (PISibs) (Waits et al., 2001). Genepop v.4.3 (Raymond & Rousset, 1995; Rousset, 2008) was used to test for deviations from Hardy-Weinberg equilibrium (HWE) and for evidence of linkage disequilibrium (LD). The Markov chain was run with 10,000 dememorisation steps, 1,000 batches and 10,000 iterations per batch. The Bonferroni sequential correction was applied to account for multiple tests (Rice, 1989).

We used the software program NewHybrids (Anderson & Thompson, 2002) to test the utility of the newly developed markers to distinguish *P. ridibundus, P. perezi,* and their hybrids. The analyses were performed using all available populations to estimate the probability of assignment of each individual to three predefined genotypic category classes: pure species 1, pure species 2, and F_1 hybrids. Since *P.* kl. *grafi* discards the whole *P. perezi* genome in its germ line before meiosis and thus only includes the unrecombined *P. ridibundus* clonal genome in the gametes, backcrosses with both parental species (and eventual F_2 hybrids, which have not been reported yet) are indistinguishable from previously defined categories (Graf et. al., 1977; Graf & Polls-Pelaz, 1989; Lodé & Pagano, 2000). Several short runs were first performed in order to detect and avoid suboptimal local maximum likelihood regions (following the authors' indications). Then a longer analysis (>2.5 million sweeps) was run. Mean assignment probability values for each individual were computed after a burn-in period of 240,000 sweeps, during which the maximum likelihood value scored in the short runs was reached. Finally, we used GenAlEx to identify private alleles diagnostic for each species by calculating allele frequencies only in individuals with concordant information at mitochondrial and nuclear sequences and microsatellites (i.e. excluding samples Rz181, Rz184, Rz143, Rz144, Rz145, Rz161, Rz162, Rz304, Rz305, Rz308, see Table 2).

RESULTS

Locus *Pper4.20* showed few alleles and was monomorphic in the population of Santo Tomé. Only one locus showed homozygote excess in one of the central Spanish populations (locus *Pper4.23* in Cerceda). According to Micro-Checker, this excess of homozygotes was generalised in many allele size classes in this population, possibly indicating the presence of null alleles rather than large allele dropout. The number of alleles ranged from 1 to 11 in Santo Tomé and from 2 to 17 in Cerceda (Table 1). Mean allelic richness was 5.38 (SE=0.59) in Santo Tomé and 8.25 (SE=1.06) in Cerceda. Observed and expected heterozygosities were generally higher in Cerceda than in

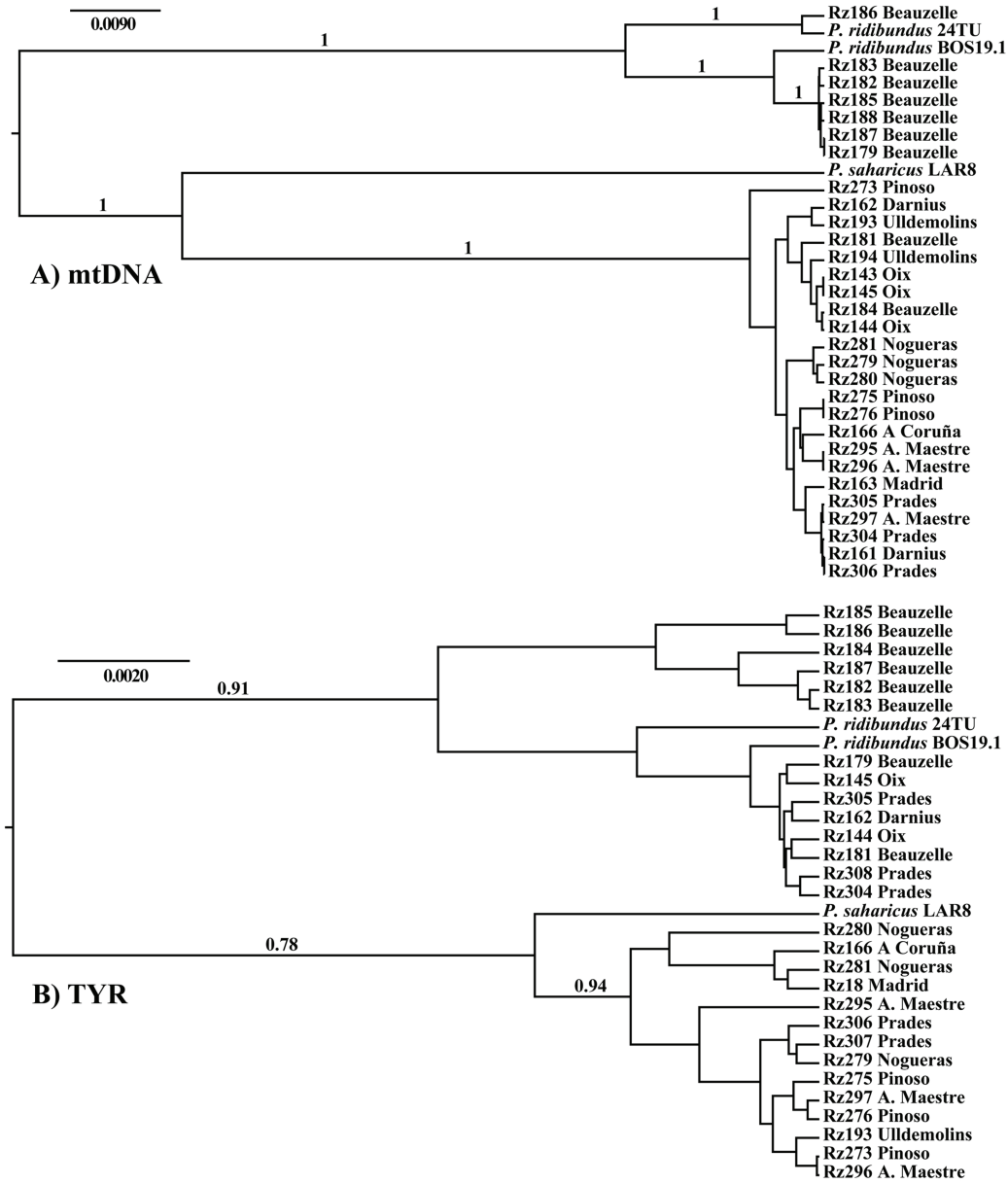


Fig. 2. Gene trees for mitochondrial (*cox1*, top) and nuclear (*TYR*, bottom) markers in *Pelophylax* samples analysed. Values on relevant nodes are Bayesian Posterior Probabilities. Sample codes as in Table 2. Scale in substitutions per site.

Santo Tomé (see Table 1). Locus *Pper4.23* in Cerceda was the only one to show significant departure from HWE after applying the sequential Bonferroni correction. Loci *Pper4.13* and *Pper4.23* were found to be consistently in linkage disequilibrium in both populations, whereas locus *Pper3.22* was in linkage disequilibrium with loci *Pper4.15* and *Pper4.7*, but only in Santo Tomé. The set of 16 loci allowed individual identification, even when accounting for possible relatives included in the sample. Moreover, just the combination of the five least informative loci was sufficient for individual recognition with 95% confidence, and seven loci were enough when accounting for relatives in the sample.

We obtained mtDNA sequences from a total of 33 individuals and nuclear (*TYR*) sequences of 31 individuals (Table 2). Gene trees were well resolved. In the mtDNA tree, fully supported clades (Bayesian Posterior Probabilities: BPPs: 1.0) included a sister-group relationship between *P. saharicus* and a monophyletic group including reference samples of *P. perezi* as well as all Iberian samples and two individuals from Beauzelle (Table 2, Fig. 2); and a clade including all the remaining samples from Beauzelle plus reference samples from Bosnia and Turkey. This "*ridibundus*" clade was further subdivided in two well-supported clades, one including one sample from Beauzelle and the reference sample from Turkey, and a second clade including the reference sample from Bosnia and the rest of the samples from Beauzelle. The *TYR* tree also recovered a sister group relationship between *P. saharicus* and *P. perezi*, although with low support (BPP: 0.78). The "*perezi*" clade included reference samples from the type locality and central Spain and all Iberian samples south of the Ebro River, as well as the individual from Ulldemolins and two individuals from Prades. The rest of the Iberian samples north of the Ebro River clustered with reference *P. ridibundus* samples

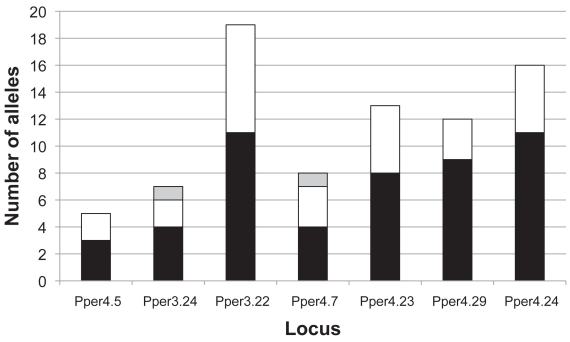


Fig. 3. Private and shared alleles found in each locus in the subset of individuals consistently assigned to *P. perezi* (*n*=13) or *P. ridibundus* (*n*=7) based on concordance between mitochondrial, nuclear and microsatellite data (see Table 2). Black bars: private *P. perezi* alleles, white bars: private *P. ridibundus* alleles, grey bars: alleles shared by both species.

and those from Beauzelle (Table 2, Fig. 2). Some samples had discordant mitochondrial and nuclear haplotypes, including two samples from Beauzelle, two samples from Oix, one from Darnius and three from Prades (Table 2). In all cases the discordance involved the presence of "*perezi*" mtDNA with "*ridibundus*" nDNA. Three of these individuals were identified as *P.* kl. *grafi* by NewHybrids, another three were classified as *P. ridibundus* with high probability (>0.9) and two additional individuals had lower assignment probabilities to *P. ridibundus* (0.83 and 0.64, Table 2). An additional individual was identified as *P.* kl. *grafi* (Rz161) based on microsatellite data. This individual had mtDNA of *P. perezi*, but unfortunately we could not amplify *TYR.* No instances of cyto-nuclear discordance were identified south of the Ebro River, where all individuals were assigned to *P. perezi* based on both mtDNA and nDNA.

Seven out of the 16 microsatellite loci cross-amplified in the samples from Beauzelle (*P. ridibundus*). The number of alleles ranged from 3 to 8. Mean allelic richness was 2.06 (SE=0.67, *n*=9). Potential null alleles were detected in loci *Pper3.22* and *Pper4.24*. Most of the 16 loci amplified in the Catalonian (Ulldemolins, Prades, Oix, Darnius) and Valencian (Pinoso, Las Nogueras, Ares del Maestre) samples (Fig. 1). Mean allelic richness estimates were 2.75 in Girona (SE=0.31, *n*=5), 3.75 in Prades (SE=0.39, *n*=5), 2.81 in Ulldemolins (SE=0.26, *n*=2), 2.81 in Ares del Maestre (SE=0.36, *n*=3), 2.31 in Las Nogueras (SE=0.27, *n*=3) and 2.63 in Pinoso (SE=0.32, *n*=3).

Only the seven markers that successfully amplified in all populations were used in the assignment analyses with NewHybrids. All samples from central Spain, Ulldemolins and south of the Ebro River were consistently assigned to one of the parental species (*P. perezi*), with probability >0.99 in all cases. All samples from France were unequivocally identified as the other parental species (*P. ridibundus*), including the two individuals with "*perezi*" mtDNA. In the populations from Oix, Darnius, and Prades, both parental species as well as some hybrids were detected. These results were mostly in agreement with data from mitochondrial and nuclear markers, although based on the distribution of diagnostic alleles (see Table 2), haplotype discordance in two individuals from Beauzelle, and in the three samples from Oix and sample Rz304 from Prades, might indicate the presence of backcrosses of *P*. kl. *grafi* with *P. ridibundus,* and of individuals of *P.* kl. *grafi* that were misclassified as pure *P. ridibundus* by NewHybrids, respectively. There was little overlap in allele frequencies across species; we identified 78 private alleles (3 to 11 per locus in *P. perezi* and 2 to 8 in *P. ridibundus*) and only two alleles shared by both species (Table 2 and Fig. 3).

DISCUSSION

The 16 newly characterised microsatellites showed moderate to high levels of polymorphism in the samples of *P. perezi* from Cerceda and Santo Tomé, and evidenced high resolution power as molecular tools in population genetic studies, even allowing individual recognition. This is essential to provide accurate estimates of genetic diversity, population structure, and gene flow in fine scale studies and to calculate effective population sizes and perform parentage analyses. Two of the markers (*Pper4.13* and *Pper4.23*) were consistently in linkage disequilibrium. Comparison of the original contigs (see GenBank Accessions) reveals very high similarity, suggesting they in fact correspond to a single locus. However, *Pper4.23* cross-amplified in *P. ridibundus* samples, whereas *Pper4.13* did not. Therefore, we decided to report results from both markers, although it is advisable to exclude *Pper4.23*, which showed null alleles in some samples, when studying *P. perezi* or klepton *grafi*.

Additionally, these new microsatellites can also help clarify unresolved issues in the *P. perezi* x *P. ridibundus* hybridogenetic complex. Most existing genetic studies on the *P.* kl. *grafi* system are based on allozyme data (Crochet et al., 1995; Lodé & Pagano, 2000; Pagano et al., 2001a, b; Schmeller et al., 2007). Microsatellite-based studies can reveal more genetic diversity than allozymes (Hotz et al., 2001) and do not require euthanising animals. Monomorphic discriminative markers may be employed to differentiate between the three taxa, but polymorphic microsatellites can reveal fine scale reproductive interactions. They thus provide better tools to trace the origin and frequency of hybridisation and introgression events and to identify taxa in the complex and delineate their respective ranges. Field discrimination between *P. perezi*, *P. ridibundus* and *P.* kl. *grafi* is challenging. Morphological characters based on the shape of vomerine teeth, the extent of interdigital membranes and some morphometric characters distinguishing each taxon (Crochet et al., 1995) are used in some field guides (Rivera et al., 2011; Ferrer & Filella, 2012). However, some of these meristic characters have overlapping ranges across species and so they are not fully discriminant. In addition, no diagnostic characters consistently differentiating species have been identified in tadpoles. Dubious reports of *P. ridibundus* in Catalonia are probably related to identification problems (Rivera et al., 2011). The use of molecular tools is thus essential for

species assignment and subsequent range delimitation within this complex.

The subset of seven microsatellite loci that cross-amplified in all samples was useful for the assignment of individuals to the three taxa in the complex. In general, we obtained high assignment probabilities and those assignments were, in most cases, in concordance with independent mitochondrial and nuclear data (see Table 2). All the Iberian samples south of the Ebro River were identified as pure *P. perezi* by the three independent molecular marker sets. Results of NewHybrids were consistent with species identification based on sequences of the nuclear marker *Tyr*, including three samples identified as *P.* kl. *grafi* by NewHybrids that had *Tyr* haplotypes characteristic of *P. ridibundus* but mtDNA of *P. perezi* and are thus either hybrids or backcrosses (Rz162 from Darnius, and Rz305 and Rz308 from Prades, Table 2). Other instances of cyto-nuclear discordance identifying individuals as hybrids or backcrosses include two individuals from Beauzelle (Rz181 and Rz184) that were consistently assigned to *P. ridibundus* but had mtDNA characteristic of *P. perezi*, and three Iberian individuals assigned with uncertainty to *P. ridibundus* (samples Rz144-145 from Oix and Rz304 from Prades, see Table 2). While based on our limited dataset it would be preliminary to identify taxon-diagnostic alleles, it is worth noting that only in the inferred area of hybridisation between the Ebro Delta and the southern slopes of the Pyrenees, private alleles of both parental species appear simultaneously in four putative *P.* kl. *grafi* individuals and in four additional specimens assigned with low probability to *P. ridibundus* (in Oix and Prades, see Table 2). All these individuals have *P. perezi* mitochondrial DNA, suggesting they are either hybrids or backcrosses and indicating that the hybrids might originate preferentially from matings between *P. ridibundus* or *P.* kl. *esculentus* males and *P. perezi* females, perhaps for behavioural reasons. All these eight samples show some alleles that are not found in any of the individuals that are consistently assigned to either of the parental species (see Table 2). These alleles are found mostly in locus *Pper3.22*, but also in *Pper4.23* and *Pper4.29*.

It should be noted that our reference sample for *P. ridibundus* (Beauzelle, near Toulouse) is geographically close to population 45 in Pagano et al. (2001a, b). These authors found both *P. ridibundus* (considered allochthonous to this region) and *P.* kl. *grafi* in this location. Our sample included two individuals with nuclear (*TYR* and microsatellites) *P. ridibundus* genotypes but *P. perezi* mtDNA (Rz181 and Rz184, Table 2). These individuals are either *P.* kl. *grafi* that NewHybrids failed to identify as such, or *grafi-ridibundus* backcrosses (or perhaps F_2 hybrids, which have not been reported yet in *P.* kl. *grafi* although there are some records in *P.* kl. *esculentus*, see Hotz et al., 1992). Lack of *P. perezi* individuals in this location both in Pagano et al. (2001a, b) and in our current work could reflect sampling biases but also its displacement by invasive *P. ridibundus*. Furthermore, Pagano et al. (2001a, b) reported that some of the *P. ridibundus* individuals analysed at this location carried a rare allele (MPI-*j*), which occurs in minor to moderate frequency in *Pelophylax* populations from Anatolia (see Pagano et al., 1997). This is consistent with our finding of very similar mtDNA haplotypes in one of the samples from Beauzelle (Rz186) and the reference sample from Turkey (24TU) (see Fig. 2). Further testing of these new markers with additional samples of *P. ridibundus* across its extensive range, as well as in related taxa, will help refine our preliminary assessment of potential species-diagnostic alleles and aid in the tracking of sources of introductions as well as in studies on the outcome of processes of interspecific hybridisation (Holsbeek & Jooris, 2010; Luquet et al., 2011).

In Catalonia, previous studies have reported the presence of *P.* kl. *grafi* along two major river basins, Ebro and Segre, as well as in the Llobregat Delta and other coastal areas (Alt and Baix Empordà, Arano & Llorente, 1995; Rivera et al., 2011; Ferrer & Filella, 2012). Our data confirm the Alt Empordà records (Darnius), and extend the presence of *P.* kl. *grafi* to the neighbouring region of La Garrotxa (Oix, in the Llierca basin, although microsatellite data were not conclusive in this case) and in upper reaches of the Llobregat River (Prades), suggesting a more widespread presence of the klepton along this river. Lack of evidence for *P.* kl. *grafi* individuals in the population of Ulldemolins, north of the Ebro River, may represent a sampling artefact (*n*=2), since it is located within the known range of *P.* kl. *grafi* (Fig. 1). On the other hand, their absence in this population could also be the consequence of a fragmented distribution, as a result of ecological and/or anthropic factors.

Previous studies have reported *P.* kl. *grafi* hybrids in southern France and in north-eastern Spain, north of the Ebro River and along its course. There are records in Catalonia, Basque Country, Navarre and Zaragoza, suggesting that *P.* kl *grafi* hybrids and/or their parental species may have crossed the Pyrenees through two routes, at the eastern and western ends of these mountains. However, it is unclear whether the hybrid complex originated in France or in Iberia, with subsequent dispersal across the Pyrenees, or independently in both regions. In addition, it is still unknown whether the *P. ridibundus* genome entered the complex from native or introduced *P. ridibundus* populations or even from *P.* kl. *esculentus* hybrids. The newly characterised microsatellites, along with other markers, will help address these questions.

The combination of mitochondrial and the newly developed nuclear markers has proven useful for species assignment and will help test hypotheses about the origin and evolutionary history of hybrid lineages. Our preliminary results suggest that the new microsatellites are useful to distinguish between the two pure lineages of coexisting waterfrogs, *P. ridibundus* and *P. perezi*, as well as the hybrid form *P.* kl. *grafi*, even when sample sizes are low. These markers can be also used to perform demographic and phylogeographic studies in *P. perezi* and are thus a valuable tool for evolutionary and conservation studies.

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