# *Genetic evidence for a distinct* Pelodytes *lineage in southwest Portugal: implications for the use of pre-developed microsatellite markers*

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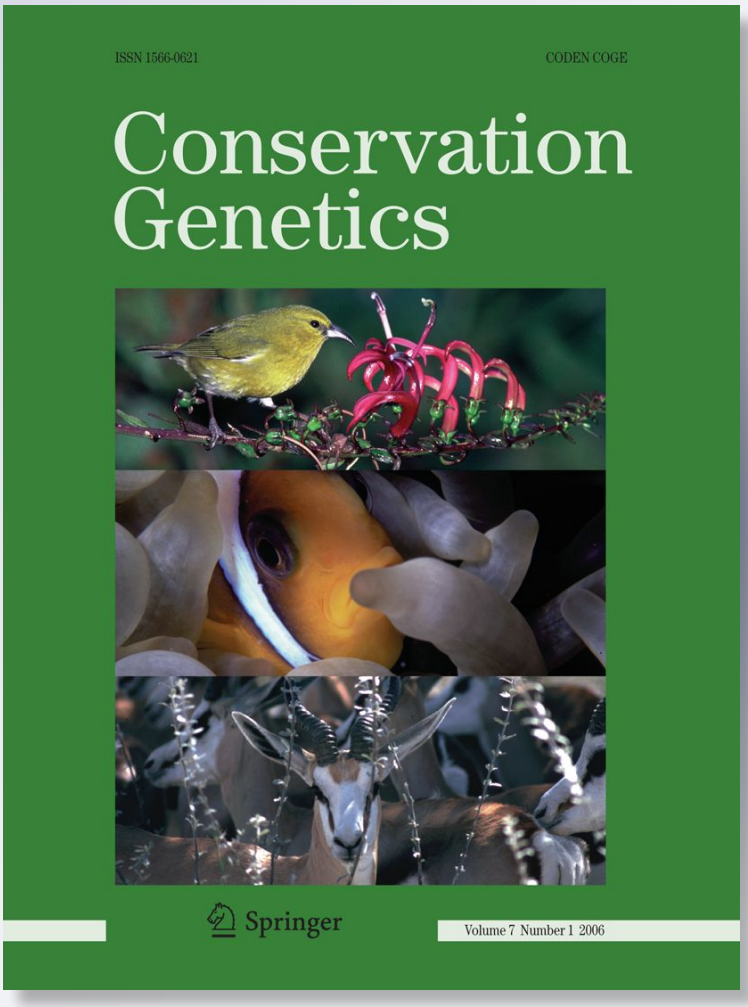


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SHORT COMMUNICATION

# Genetic evidence for a distinct *Pelodytes* lineage in southwest Portugal: implications for the use of pre-developed microsatellite markers

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**Abstract** For population genetic analyses of Parsley frogs in Iberia we initially used microsatellite markers previously developed for *Pelodytes punctatus* in southwest Portugal. However, several loci indicated a strong signal of amplification failure for individuals from northern Spain. Cryptic species or genetic entities are suspected to occur in southwest Portugal on the basis of studies with other species and understanding cryptic diversity is a concern as amphibian habitat, temporary ponds in traditional Mediterranean farmland, is disappearing at a fast rate. Our study revealed a new *Pelodytes* lineage in southwest Portugal which appears to be discrete. However, the rare occurrence of a distinct mitochondrial haplotype from its sister species, despite no nuclear differentiation, is a signature of introgression indicating that reproductive isolation is not complete.

**Keywords** Biodiversity · Fine-scale · Microsatellite amplification failure · Organelle introgression · *Pelodytes* · Southwest Portugal

**Electronic supplementary material** The online version of this article (doi:10.1007/s10592-011-0299-5) contains supplementary material, which is available to authorized users.

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## Introduction

Parsley frogs (*Pelodytes punctatus*, Daudin 1803) inhabit large areas in western Portugal, northern, central and eastern Spain, most of France, and in coastal northwestern Italy. The Iberian Parsley frog *P. ibericus* (Sánchez-Herráiz et al. 2000) is a morphologically similar sister species of *P. punctatus*, with a range restricted to south-eastern Portugal and south-western Spain. The time of divergence between the two is still not completely resolved due to disputed *P. punctatus* lineages, but is likely to be near the Pliocene–Pleistocene boundary (García-París et al. 2003; Veith et al. 2006). Only *P. punctatus* is supposed to have expanded its range into northern areas post-glacially (e.g. Gasc et al. 1998).

Although *P. punctatus* is widely distributed, it has a disjunct distribution between Portugal and the other areas in Iberia and elsewhere (Fig. 1). A possible distinct lineage of *Pelodytes* endemic in southwest Portugal has been suggested (e.g. Paillete et al. 1992; Crespo et al. 2010). This is supported by recent observations that microsatellites developed for *P. punctatus* using individuals from southwest Portugal (Van de Vliet et al. 2009) show strong amplification failure for individuals ($N = 17$) from northern Spain (ca. 25 ± 21% average failure for 14 loci plus possible null-alleles at seven loci, see Supplementary Table S1). Normally we find 0.3–0.5% failure across all 14 loci for individuals from southwest Portugal (unpublished data). Cross amplification failure indicates divergence between these groups as found in other amphibians (e.g. Hendrix et al. 2010). This cross-amplification failure between the distinct populations of *P. punctatus* is comparable and even higher than for the sister species *P. ibericus* originating from neighbouring east Algarve and Alentejo, Portugal. For this species, the 14 loci show an

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average failure of ca. 17 ± 22%, plus possible null-alleles at four loci (Supplementary Table S1). For each locus amplification products are near the size range as observed for individuals from southwest Portugal, except for results from tree and five loci for *P. ibericus* and *P. punctatus* from northern Spain, respectively, revealing unique alleles of in general larger sizes (Supplementary Table S1). These patterns of divergence are also consistent with the view that the geographical and topographical heterogeneity of this part of the Iberian Peninsula have played an important role as refugia within the Iberian Peninsula during Pleistocene climatic changes (Gómez and Lunt 2007). These geographical constraints influenced (sub-)speciation processes creating distribution patterns coincident for several other vicariant amphibian (sub)species (e.g. Martínez-Solano et al. 2006; Gonçalves et al. 2007).

Resolving the phylogenetic status of *Pelodytes* from southwest Portugal has implications to assess its conservation status and to prioritize conservation actions. Indeed, although the overall status of both *P. punctatus* and *P. ibericus* has been considered of least concern by IUCN (Bosch et al. 2008; Denoël et al. 2008), the *Pelodytes* lineage from southwest Portugal may be of a greater concern due to its relatively restricted range. Furthermore, this *Pelodytes* mostly breed in temporary ponds associated with low-intensity farming systems (Crespo et al. 2010), which are rapidly disappearing due to ongoing trends for agricultural intensification (Beja and Alcazar 2003). The presence of a genetically distinct lineage will also imply that the published microsatellite loci for *P. punctatus* (Van de Vliet et al. 2009) are of limited use for *P. punctatus* in northeast Iberia.

This study addressed whether *Pelodytes* in southwest Portugal (hereafter coded as *P. sp?*) is genetically different compared to *P. punctatus* from other regions, thus explaining the detected microsatellite amplification failure. Analyses were based on mitochondrial cytochrome *b* and 16S rRNA fragments, which have proved to be useful tools to solve disputed taxonomic issues in amphibian species complexes including *Discoglossidae* and *Pelobatoideae* (e.g. García-París et al. 2003; Fromhage et al. 2004; Veith et al. 2006). Analysis based on nuclear microsatellite data (van de Vliet et al., unpublished) were also used to complement mitochondrial-based inferences, as the organelle genomes are particularly prone to introgressions and sweeps, resulting in phylogenies that are distinct from the actual phylogeny of the species, particularly in parapatric sister taxa with uncertain levels of reproductive isolation (e.g. Funk and Omland 2003).

## Materials and methods

The *Pelodytes* species that occur in southern Portugal (Algarve and Alentejo) were sampled from multiple ponds ($n_{ponds} = 11$ and 24 within the range of *P. ibericus* and *P. sp?* respectively, Fig. 1) with at least five individuals per sample site (breeding pond). In addition, 1–4 individuals of *P. punctatus* from one site in Northern Spain (El Musco Lagardia Araba) were included in our analyses. For all sites

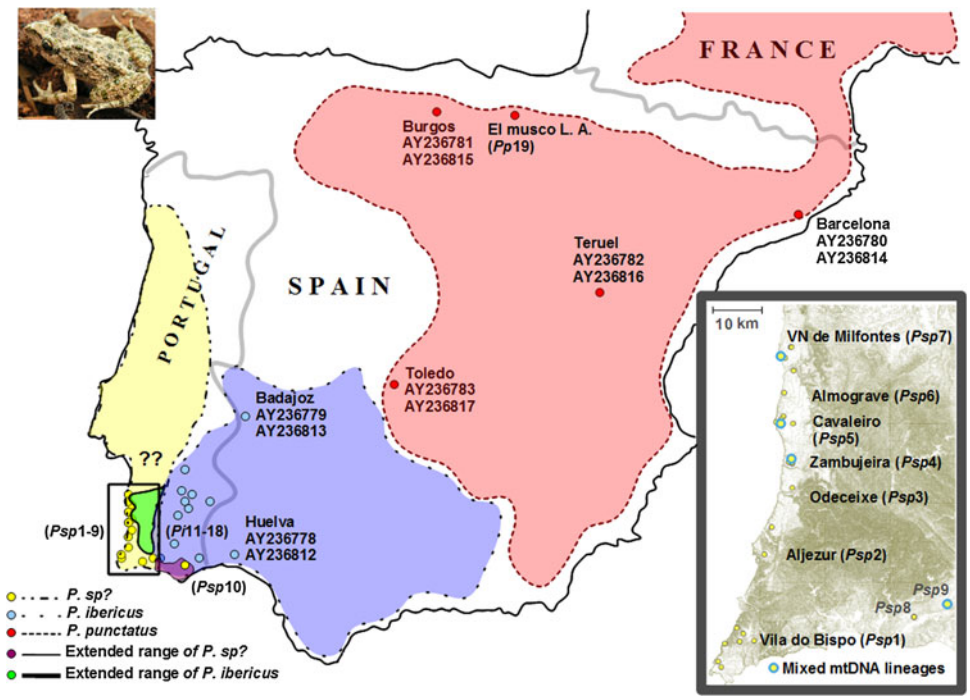


**Fig. 1** Distribution of *Pelodytes punctatus* and *Pelodytes ibericus* in the Iberian Peninsula and southern France (redrawn from Bosch et al. 2008; Denoël et al. 2008). *Pelodytes* individuals found in southwest Portugal (which were supposed to be *P. punctatus* are coded as *P. sp?*). Locations of collected or published samples (AY- numbers, GenBank) are illustrated and in brackets the corresponding ID-location as described in Appendix A. The extended ranges are possible contact zones between *P. sp?* and *P. ibericus*

where a mixture of the two *Pelodytes* lineages was suspected from the initial mitochondrial results, additional individuals and breeding ponds were included and sampled within the same year. Sampling involved mainly taking tail clips of tadpoles and toe clips from two adult frogs. DNA was extracted following a standard phenol–chloroform extraction protocol (Sambrook et al. 1989). We amplified fragments coding for a 331 bp portion of cytochrome *b* and a 580 bp portion of 16S rRNA mitochondrial genes. Primers for Cytochrome *b* were newly designed using sequences from *P. punctatus* and *P. ibericus* (GenBank AY236778-83) (annealing temperature $T_a = 55°C$):

Cyt *b* *Psp*Forward: 5′ AGCTCACCCTCTGACAAAAA TTATAAACGA 3′
Cyt *b* *Psp*Reverse: 5′ GGGTAGGAGGTAGCCTATGA AAGC 3′

The primers '16Sar' and '16Sbr' (Palumbi et al. 1991) ($T_a = 48°C$) were known to successfully amplify *Pelodytes* spp. (García-París et al. 2003; Veith et al. 2006). PCR amplifications were performed in a 25 µl reaction volume containing approximately 20 ng DNA, 0.5 µM of each primer, 2.0 mM $MgCl_2$, 5× Go*Tag* Flexi buffer (Promega), 0.2 mM of each dNTP and 1.0 U Go*Tag* DNA Polymerase (Promega). The PCR program held 38 cycles with 95°C for 40 s, primer specific annealing temperature for 60 s, and 72°C for 60 s. For all PCR reactions we started with a denaturation step of 94°C for 5 min and the last cycle was followed by a 7 min extension at 72°C.

Alignments of nucleotide sequences were constructed with Geneious 4.8.5 (Drummond et al. 2009), including 4 published sequences (GenBank) of *P. punctatus* from a wide range in Spain (cytochrome *b*: AY236780-83; 16S rRNA: AY236814-17) and two published sequences of *P. ibericus* from southeast Spain (cytochrome *b*: AY236778-9; 16S rRNA: AY236812-13). In addition, a sequence similarity search was performed to confirm the taxonomic status (Appendix) and the average number of nucleotide differences was defined using DnaSP 4.1 (Rozas 2009). For subsequent analyses the two partial mitochondrial sequences were combined into a single data set (909 bp) only for individuals having distinct haplotypes and when both sequences were available. In addition, sequences of two outgroup species were included in the alignment: *P. caucasicus* (Cytochrome *b* AY236777; 16S rRNA MVZ218724, GenBank) and *P. cultripes* (Cytochrome *b* JF272532-33, 16S rRNA JF275860-61, GenBank).

Phylogenetic relationships were estimated using (ML) Maximum Likelihood (PhyMLv2.4.4; Guindon and Gascuel 2003) and (BI) Bayesian inference (MrBayes version 3.1; Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The Akaike Information Criterion (AIC) implemented in jModeltest version 0.1.1 (Posada 2008) revealed that the best fit model of sequence evolution was GTR + Gamma. This was used for ML and Bayesian analyses. For ML we applied the estimated transition/transversion ratio and proportion of invariable sites. In the Bayesian analyses default settings were used for the prior distribution. We ran four Metropolis coupled Monte Carlo Markov chains (MC3) for 1.100.000 generations, sampling every 200 generations and 500 trees as burn-in were discarded. Robustness of the inferred trees was evaluated by bootstrapping (1,000 replicates) and using Bayesian posterior probabilities.

Clustering patterns for individuals of *P. sp?* ($N = 461$) in relation to *P. punctatus* ($N = 17$) and *P. ibericus* ($N = 15$) were analysed using data from 14 microsatellite loci (van de Vliet et al. 2009) and applying factorial correspondence analysis (FCA, Genetix 4.05, Belkhir et al. 2000). In addition, three breeding sites where we found multiple mitochondrial lineages were further compared with individuals from breeding sites with single lineages of *P. sp?* and *P. ibericus*. For details of used PCR conditions per microsatellite locus see van de Vliet et al. (2009). Differences in membership proportion for individuals with distinct mitochondrial lineages were, in addition, determined by applying a Bayesian clustering approach implemented in the software Structure 2.3.1 (Pritchard et al. 2000). Genetic units are defined by minimizing linkage disequilibrium (LD) and departures from Hardy–Weinberg equilibrium (HWE). Ten independent runs with 100.000 iterations and a burn-in of 25.000 were performed; assuming admixture, correlated allele frequencies and without a priori spatial information of individuals. After defining the appropriate value of lambda, the best number of genetic units (K) was determined by comparing the log-likelihood considering K between 1 and 6 and subsequently by computing ΔK (Evanno et al. 2005).

## Results and discussion

### Genetic relationships and divergence

All haplotypes were deposited in GenBank: cytochrome *b* (JF272534-42) and 16S rRNA gene (JF272520-30). Most individuals from southwest Portugal could not be assigned (>99%) to either *P. punctatus* or *P. ibericus* (Appendix). For example, cytochrome *b* sequence data indicated a relative low sequence similarity score of 96.4–97.7% and 96.1–96.4% (average number of nucleotide differences >14.5 and 8.9) for *P. punctatus* and *P. ibericus*, respectively. Exceptions were several individuals found in four unique sites, each located in a different region (*Psp*4, 5, 7 and 9). The haplotypes for these individuals highly matched (>99.5%) with published sequences of *P. ibericus*, indicating a mixture of taxa (Appendix).

Phylogenetic analyses detected well-supported groups within *Pelodytes* (Fig. 2). Individuals from southeast Portugal and northern Spain clustered with published sequences of respectively *P. ibericus* and *P. punctatus*, confirming their expected taxonomic status, despite the suggestion that *P. ibericus* could be paraphyletic (using only cytochrome *b* data *P. ibericus* seems monophyletic, results not shown). Most mitochondrial sequences from *P. sp?* cluster as a sister lineage to *P. punctatus* whereas others cluster within *P. ibericus*, suggesting that a mixture of two species might be present in samples of *P. sp?* (Fig. 2). Nuclear analyses based on fourteen microsatellites revealed that there was no differentiation between individuals of *P. sp?* containing distinct mitochondrial lineages. Two of the three dimensions of a Multivariate Factorial Correspondence analysis (FCA) accounted for all of the variability, illustrating three clearly genetically distinct *Pelodytes* lineages with no further subdivision within *P. sp?* (Fig. 3a). STRUCTURE (minimizing Hardy–Weinberg and Linkage Disequilibria) clustered individuals according to the ponds they were sampled from, not their distinct mitochondrial genomes, thereby revealing nuclear similarity and absence of reproductive barriers between the individuals with distinct organelle types (Fig. 3b).

*P. sp?* shows a very restricted southwest distribution in Europe (Fig. 1), with relatively low nuclear differentiation (Fig. 3a). Furthermore, a large group of individuals share almost identical mitochondrial haplotypes and accounted for a small part of the total genetic variation (Fig. 2). The confined phylogeographic distribution of *Pelodytes* in Portugal and several other vicariant amphibian (sub)species (*Alytes cisternasii, Discoglossus galganoi, Lissotriton boscai, Salamandra salamandra crespoi*) may probably be explained by contractions of populations during the Last Glacial Maximum (Fromhage et al. 2004; García-París et al. 2003; Martínez-Solano et al. 2006; Veith et al. 2006). We argue that there is support for *P. sp?* having a highly restricted post-glacial recolonization, rather that being the founder for *P. punctatus* in the central and northeastern range of the Iberian Peninsula.

## *P. sp?* range in relation to *P. ibericus*

Sites (ponds) with mixed haplotypes (*P. ibericus* and *Psp?*), most likely as a result of hybridization, were found in: Vila Nova de Milfontes (*Psp*7), Zambujeira (*Psp*4), Cavaleiro (*Psp5*) and Nave do Barão (*Psp*9) (Fig. 1). Microsatellite data (14 loci) did not support interspecific hybridization in these areas since no individuals were characterized by the presence of *P. ibericus*-specific alleles and there was no genetic differentiation ($P > 0.05$) between individuals with the distinct haplotypes within breeding sites. The lack of microsatellite support for hybridization is visualized in the FCA and STRUCTURE analyses, where individuals from each mixed site cluster together in relation to other sites instead of clustering according to their species-specific haplotypes for all mixed sites (Fig. 3b, *Psp*9 was not included in the analyses). After defining the best number of genetic units ($K = 2$) it would still not divide according to organelle types.

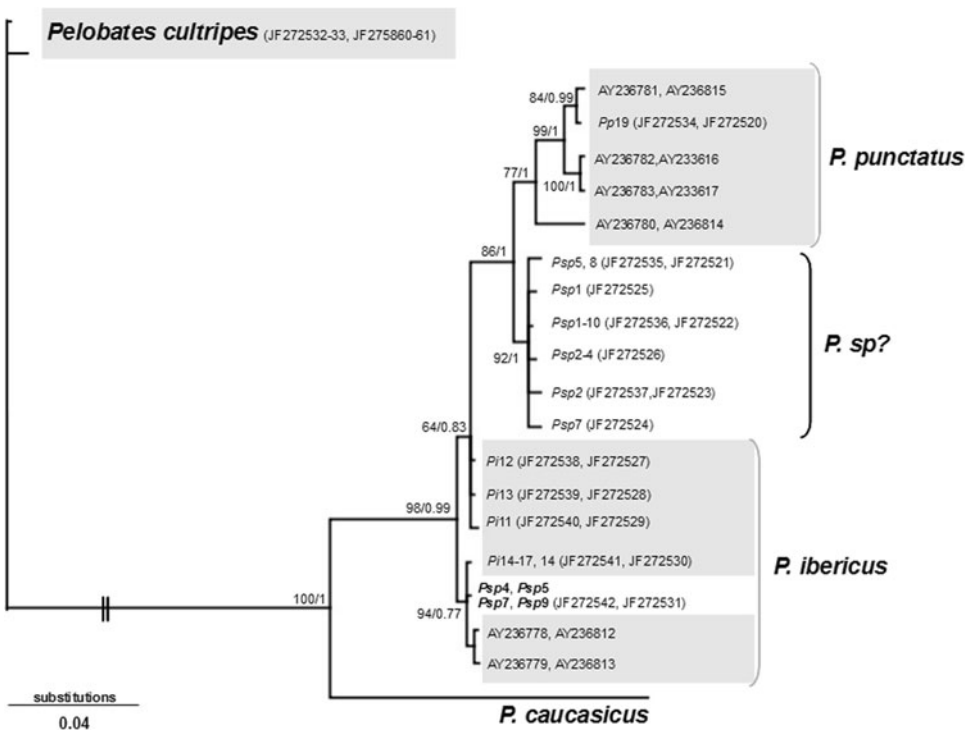


**Fig. 2** Genetic relationships of *Pelodytes* inferred from partial sequence data of the mitochondrial cytochrome *b* and the ribosomal 16SrRNA genes. Numbers in the major nodes correspond to maximum likelihood (*left*) and BI posterior probabilities (*right*). ID-location and cytochrome *b* and 16S associated accession number (GenBank) are shown. When haplotypes for locations in southwest Portugal highly matched with published sequences of *P. ibericus*, they are illustrated in *bold*

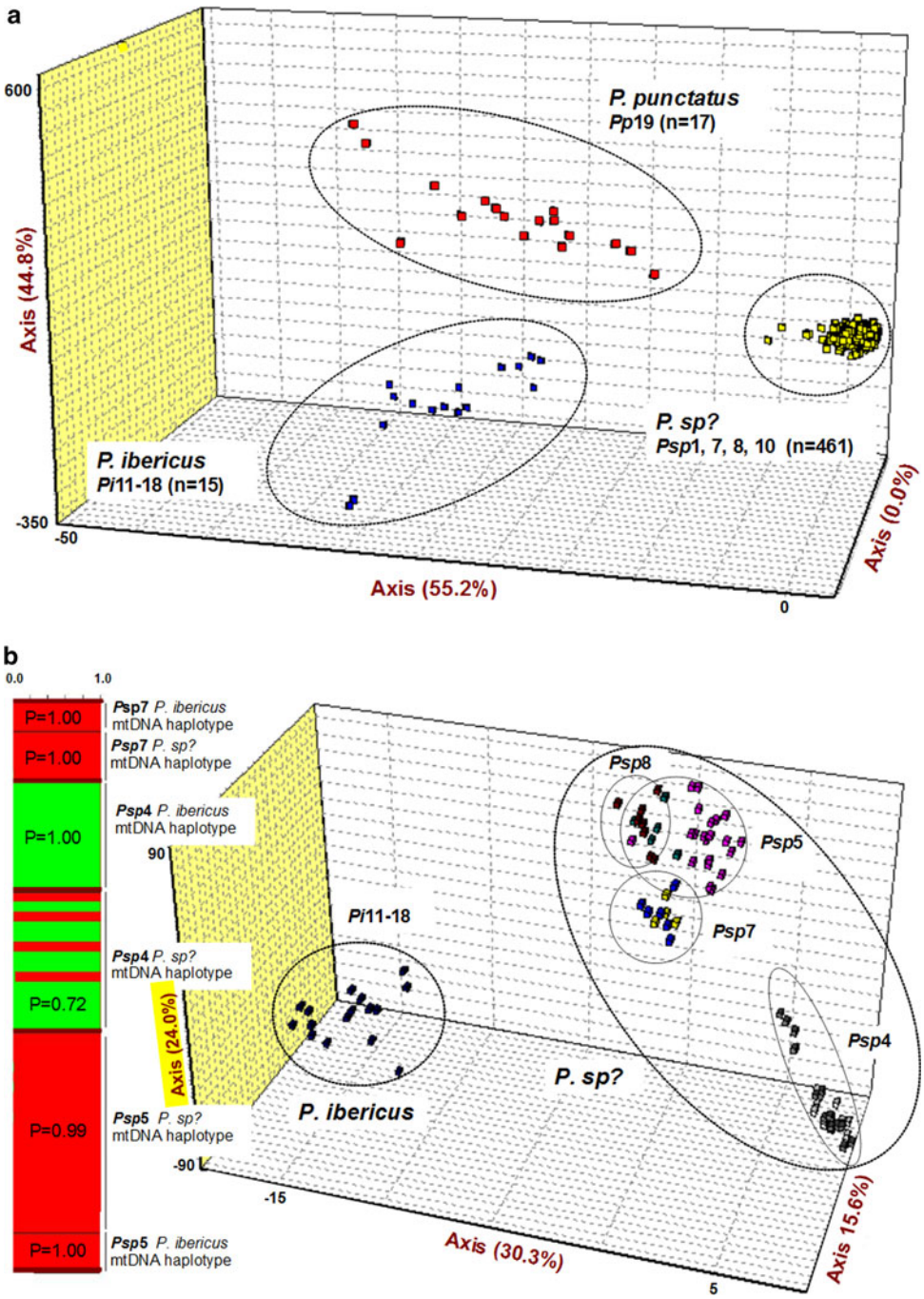


**Fig. 3** Differentiation with microsatellites **a** Factorial Correspondence analysis (FCA) for two confirmed *Pelodytes* species *P. punctatus* (*Pp*19) and *P. ibericus* (*Pi*11-18) and individuals of *Pelodytes sp.* (*Psp*?) from southwest Portugal **b** and Structure analysis (K=2) for individuals from breeding sites with mixed mitochondrial haplotypes between *Pelodytes sp.* and *Pelodytes ibericus* (*Psp*, 5 and 7). In the FCA individuals with distinct haplotypes per breeding site are illustrated as squares with two different colours. *Psp* is not included since it only had one individual with a *P. ibericus* haplotype. For comparison reasons individuals from sites showing no mixed mitochondrial haplotypes were included in the FCA analyses (*Psp*8 *Psp?*; *Psp*11-18 *P. ibericus*). in the bar plot are orientated according to their breeding site (*Psp*7, 4 and 5 from top to bottom) and to their mitochondrial haplotype (mtDNA) found within these breeding sites. For ten independent runs the average membership proportion (P) to the assigned cluster are almost equal, standard deviation ≤0.001

This indicates that, although hybridization is the likely cause for the presence of *P. ibericus* organelle lineages in a few sites within the *P. sp?* range, it left no detectable traces in the nuclear genome which is a signature of introgression. Unfortunately, we have no data as to whether overlapping ranges occur in areas more north and centrally located in Portugal and which possibly contained the ancestral lineage of *P. ibericus* which gave raise to the hybridized populations.

Our study has revealed the presence of a distinct *Pelodytes* lineage in southwest Portugal, explaining amplification failure of several microsatellite loci in *P. punctatus* and *P. ibericus*. Poor knowledge of contact zones with possible introgressive hybridization and high intraspecific diversity observed for *Pelodytes* lineages (e.g. García-París et al. 2003) indicate that the phylogeographic history remains unclear and further studies are necessary to resolve a complete molecular phylogenetic tree for the *Pelodytes* genus. Given that suitable breeding habitat in this region is disappearing at an alarmingly fast rate (Beja and Alcazar 2003; van de Vliet et al. unpublished) affecting overall Mediterranean biodiversity (Stoate et al. 2009), it is urgent to

establish conservation status and conservation measures for *Pelodytes* in southwest Portugal.

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## Appendix

See Table 1.

**Table 1** Sample location and ID, sample sizes for mitochondrial data and results of species similarity search (GenBank)

| Location | ID | Number of sampled breeding sites or ponds//total number of individuals (N)[a] | Similarity score (>99%) with published and accepted *Pelodytes* species (– = identification failure) | Species assignation |
|---|---|---|---|---|
| (west) Algarve: Sagres, Vila do Bispo | *Psp*1 | 6//28–29 | – | *Pelodytes* sp. *(P. sp?)* |
| (west) Algarve: Aljezur, Rogil | *Psp*2 | 2//9–10 | – | *Pelodytes* sp. *(P. sp?)* |
| (west) Alentejo: Odeceixe | *Psp*3 | 1//7–9 | – | *Pelodytes* sp. *(P. sp?)* |
| (west) Alentejo: Zambujeira | *Psp*4 | 4//25–34 | –, 1 breeding site with *P. ibericus* haplotype | *Pelodytes* sp. *(P. sp?)* |
| (west) Alentejo: Cavaleiro | *Psp*5 | 3–4//26–40 | –, 1 breeding site with *P. ibericus* haplotype | *Pelodytes* sp. *(P. sp?)* |
| (west) Alentejo: Almograve | *Psp*6 | 2//11–14 | – | *Pelodytes sp. (P. sp?)* |
| (west) Alentejo: Vila Nova (VN) de Milfontes | *Psp*7 | 2–3//13–28 | –, 1 breeding site with *P. ibericus* haplotype | *Pelodytes* sp. *(P. sp?)* |
| (southwest) Algarve: Tunes | *Psp*8 | 1//8 | – | *Pelodytes* sp. *(P. sp?)* |
| (south) Algarve: Nave do Barão | *Psp*9 | 1//8 | –, 1 breeding site with *P. ibericus* haplotype | *Pelodytes* sp. *(P. sp?)* |
| (south) Algarve: Quelfes | *Psp*10 | 1//5 | – | *Pelodytes* sp. *(P. sp?)* |
| (east) Algarve: Castro Marim | *Pi*11 | 1//1 | *P. ibericus* | *P. ibericus* |
| Alentejo: Mértola—Horta do Tio Luís | *Pi*12 | 1//1 | *P. ibericus* | *P. ibericus* |
| Alentejo: Azinhal | *Pi*13 | 1//1 | *P. ibericus* | *P. ibericus* |
| Alentejo: Mértola—Guerreiro-S. Marcos | *Pi*14 | 1//1 | *P. ibericus* | *P. ibericus* |
| Alentejo: Monte das Figueiras | *Pi*15 | 2//2 | *P. ibericus* | *P. ibericus* |
| Alentejo: Aljustrel | *Pi*16 | 2//2 | *P. ibericus* | *P. ibericus* |
| Alentejo: Odavelas | *Pi*17 | 1//5 | *P. ibericus* | *P. ibericus* |
| Alentejo: Serpa | *Pi*18 | 2//2 | *P. ibericus* | *P. ibericus* |
| El musco Lagardia Araba (north Spain) | *Pp*19 | 1//1–4 | *P. punctatus* | *P. punctatus* |

[a] Number of sites and individuals vary depending on the obtained sequence data used for analyses

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