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A simplified molecular method for distinguishing among species and ploidy levels in European water frogs (*Pelophylax*)

J. SUSANNE HAUSWALDT,* MANUELA HÖER,* MARIA OGIELSKA,† DITTE G. CHRISTIANSEN,‡ DARIA DZIEWULSKA-SZWAJKOWSKA,† ELŻBIETA CZERNICKA† and MIGUEL VENCES*
Zoological Institute, Technical University Braunschweig, Mendelssohnstr. 4, 38106 Braunschweig, Germany, †Department of Evolutionary Biology and Conservation of Vertebrates, University of Wrocław, Wrocław, Poland, ‡Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland

Abstract

Western Palearctic water frogs in the genus *Pelophylax* are a set of morphologically similar anuran species that form hybridogenetic complexes. Fully reliable identification of species and especially of hybrid ploidy depends on karyological and molecular methods. In central Europe, native water frog populations consist of the *Pelophylax esculentus* complex, that is, *P. lessonae* (LL), *P. ridibundus* (RR) and the hybrid form *P. esculentus* that can have different karyotypes (RL, LLR and RRL). We developed existing molecular methods further and propose a simple PCR method based on size-differences in the length of the serum albumin intron-1 and the *Rana*CR1, a non-LTR retrotransposon of the chicken repeat (CR) family. This PCR yields taxon-specific banding patterns that can easily be screened by standard agarose gel electrophoresis and correctly identify species in all of the 160 samples that had been identified to karyotype with other methods. To distinguish ploidy levels in LR, LLR and RRL specimens, we used the ratio of the peak heights of the larger (*ridibundus* specific) to the smaller (*lessonae* specific) bands of fluorescently labelled PCR products resolved on a capillary DNA sequencer and obtained a correct assignment of the karyotype in 93% of cases. Our new method will cut down time and expenses drastically for a reliable identification of water frogs of the *P. esculentus* complex and potentially for identification of other hybridogenetic complexes and/or taxa, and it even serves as a good indicator of the ploidy status of hybrid individuals.

Keywords: hybridization, *Pelophylax* (*Rana*) *lessonae*, *Pelophylax esculentus*, *Pelophylax ridibundus*, polyploidy, serum albumin

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Introduction

To detect hybrid individuals from their parental taxa, molecular tools are often the only choice and methods such as restriction enzyme chromosome banding (Leitão *et al.* 2007), or high-throughput approaches such as multiplex PCR of both mtDNA and nuclear DNA genes followed by a RFLP analysis (e.g. Prado *et al.* 2011), AFLP (e.g. Congiu *et al.* 2001), or microsatellite analyses (Kothera *et al.* 2009) are being used. For detection of polyploid individuals, cytogenetic assessment of chromosome numbers, for example, via flow cytometry of DAPI or propidium iodide (PI)-stained nuclei, is performed (e.g. Nishiwaki *et al.* 2011). Allozymes can also identify chromosome copy numbers via the number of bands or the dosage of bands (reviewed in Mable *et al.* 2011). For microsatellites, however, uneven amplification of alleles during the PCR can hamper the direct assessment of ploidy status through intensity of bands (e.g. measured as fluorescent peak height), but the number of joint occurrence of—ideally parental taxon-specific-alleles—is frequently used for detection of polyploid hybrids (e.g. Julian *et al.* 2003).

Western Palearctic water frogs (WPWF) in the genus *Pelophylax* are a series of about twelve morphologically similar species of largely aquatic anurans, several of which hybridize and some even form hybridogenetic species complexes (Graf & Polls Pelaz 1989). The WPWF taxa found most commonly in central and eastern Europe belong to the *P. esculentus* complex, consisting of the Pool frog (*P. lessonae*; L, nuclear genome denoted LL), the Marsh frog (*P. ridibundus*; R, RR), as well as their interspecific hybrid, the Edible frog (*P. esculentus*; E, RL). The hybrid taxon (RL) originates from hybridization of LL and RR, and it reproduces by hybridogenesis with either parental taxon. During this process, the genome of only one parent (in most cases the R genome) is transferred to

Correspondence: J. Susanne Hauswaldt, Fax: (+49) 531 3918198; E-mail: s.hauswaldt@tu-bs.de

the gametes (usually clonally), and the genome of the other parent is discarded before meiosis (Tunner & Heppich 1981). The offspring resulting from such pairing receives a clonally inherited (from the hybrid) and a regular Mendelian style inherited (from the species) genome. Therefore, these offspring are referred to as hemiclones. We are aware that such hemiclones do not represent independent evolutionary lineages, and thus, they probably should not be recognized at the species level. For convenience, we nevertheless herein use the established name *P. esculentus* to refer to the RL hybrid and also refer to other hemiclones using the species-level names that have been proposed for them.

The RL hybrid is frequently perpetuated hemiclonally in mixed populations with one of the parent species, in central Europe most commonly with *P. lessonae* (reviewed in Graf & Polls-Pelaz 1989). Although the inheritance of one genome by the hybrid is mainly clonal, introgression has been detected in the mitochondrial (Spolsky & Uzzell 1984; Plötner *et al.* 2008) and nuclear genome (reviewed in Pagano & Schmeller 1999). However, all-hybrid populations also exist in which hybrid females often lay diploid eggs, resulting in a particularly high frequency of triploids (reviewed in Holsbeek & Jooris 2010; Czarniewska *et al.* 2011). The chromosome exclusion (mainly of the L genome) during hybridogenesis has been attributed to a large part to some unknown factors on the R genome, but interestingly, geographic differences in *P. ridibundus* exist in the ability to induce genome exclusion (reviewed in Holsbeek & Jooris 2010). *Pelophylax kurtmuelleri* originating from Southern Europe are not capable to induce hybridogenesis in hybrids with *P. lessonae* (Hotz *et al.* 1985), and the southern taxon *P.* cf. *bedriagae* (from Anatolia) is also not capable of doing this (Plötner 2005).

In the *esculentus* complex, the two parental taxa, *P. lessonae* and *P. ridibundus*, have different habitat preferences and can be distinguished morphologically. The call of *P. ridibundus* also differs clearly from that of *P. lessonae* and *esculentus* (reviewed in Plötner 2005). However, identification of the hybrid is more complicated: it is intermediate in size and other morphological characters, and its habitat preferences cover those of both parental species. Based on morphological characters, in particular the size and shape of the metatarsal tubercle, it is possible to assign 90% of adult individuals correctly to either parental or the hybrid, even in the field (Plötner 2010), but morphological features of *P. esculentus* hybrids with the same genotype composition may differ among populations (Plötner *et al.* 1994). Tadpoles of hybrids, however, cannot be identified with certainty based on their morphology (Günther 1996). The problem of identification using morphological characters is exacerbated even further by the fact that there are not only diploid but also triploid hybrids, which can resemble either parental species even more closely because of gene-dosage effects. LLR hybrids carry two sets of chromosomes of *lessonae* and one of *ridibundus* while RRL hybrids carry two sets of chromosomes of *ridibundus* and one of *lessonae*. Kierzkowski *et al.* (2011) found that three major morphological indices used in identification of water frogs based on the length of the digitus primus (DP), callus internus (CI), tibia (T) and femur (F), namely DP/CI, T/CI and F/T, allowed for correct classification of 91% of diploid hybrids, but only 84% of LLR and 52% of RRL were correctly classified.

Distinction of LL, RL and RR, respectively, LLR, RRL is possible with a combination of morphometric and erythrocyte measurements. A study with 151 LL, LLR, RL, RRL and RR, confirmed by genetic methods, showed 99% success with this erythro-morphometric method on Northern European water frogs (Christiansen 2011), but it does not work well in many Central European water frog populations (Plötner *et al.* 1994). However, the method is very time-consuming, and in addition, it requires invasively collected samples. Erythrocyte size reflects DNA content and therefore allows for reliable discrimination between diploid and triploid WPWFs (Uzzell & Berger 1975), but this method by itself does not allow reliable discrimination between LLR and RRL (Ogielska *et al.* 2004). Other approaches, such as allozyme analysis of LDH-B (Uzzell & Berger 1975, Hotz *et al.* 2008) or scoring a set of microsatellite loci (Christiansen 2009), have also been used to identify the genomic make-up, but again these methods are labour-intensive and time-consuming and allozyme analysis also requires invasive sampling. The fastest molecular approach for taxon identification that will work with noninvasively collected samples is the recently published PCR-RFLP method that consists of one PCR followed by a restriction digest (Patrelle *et al.* 2011). However, only 83% of all 93 individuals tested were assigned to the same taxon that had been inferred by allozyme analysis using four enzymes. Furthermore, this method cannot be used to distinguish between diploid and triploid hybrids.

Because of the problems with morphological identification, individuals often are not even classified to species level during habitat surveys, but only as water frogs (reviewed in Plötner 2005). This, however, poses a problem to conservation issues, in particular with respect to *P. lessonae*, the most threatened taxon in the *esculentus* complex with the narrowest habitat width. Recent and repeated introductions of non-native *P. ridibundus*, *P. bedriagae* and *P.* cf. *bedriagae* for consumption, pet trade and scientific purposes have been reported from Western and Central Europe (e.g. Hoogmoed 1975; Pagano *et al.* 2001; Vorburger & Reyer 2003; Holsbeek *et al.* 2008). During a recent genetic screening of 944 water frogs in Belgium, 79% were discovered to be not native:

while a few individuals belonged to different species (*P. perezi*, pure or hybrid, or *P. bedriagae*), most belonged to non-native haplogroups of *P. ridibundus* (Holsbeek *et al.* 2010). Because of their different capabilities of inducing hybridogenesis, consequences of introduction of *P. ridibundus* of the Central or Southern group may likely result in reduction (maybe even elimination) of *P. lessonae* and potentially in endangering *P. esculentus* (Holsbeek & Jooris 2010). However, difficulties in identification of native and introduced taxa exacerbate assessment of the current taxon composition of most populations. Therefore, a reliable and easily performed molecular method is needed that ideally also works on noninvasively derived DNA samples.

Here, we present a molecular method based on a single PCR that allows discrimination of *P. ridibundus*, *P. lessonae* and *P. esculentus*, as well as giving a good indication of the karyotype of the hybrid. This approach is based on differences in DNA sequence length of serum albumin intron-1 (SAI-1) between *P. ridibundus* and *P. lessonae* (Plötner *et al.* 2009). RanaCR1 is approximately 550 bp shorter in *P. lessonae* than in *P. ridibundus*. (Fig. 1 and Table 1).

Materials and methods

Based on the published sequences of serum albumin intron-1 for *Pelophylax ridibundus* (Genbank accession numbers FN432363–67, FN432371) and *P. lessonae* (FN432381–85) from Plötner *et al.* (2009), we designed new primers that would encompass the section of the CR1 that is shorter in *P. lessonae* and should therefore generate PCR products of different length for each species, as well as both products in hybrids. The primers Pel-SA-F1 (5′ TCCATACAAATGTGCTAAGTAGGTT-3′) and Pel-SA-R2 (5′ GACGGTAAGGGGACATAATTCA 3′) bind in the intron-1 i.e., to the sequence of *P. ridibundus* (FN432364) at positions 35–59 and 854–875, respectively.

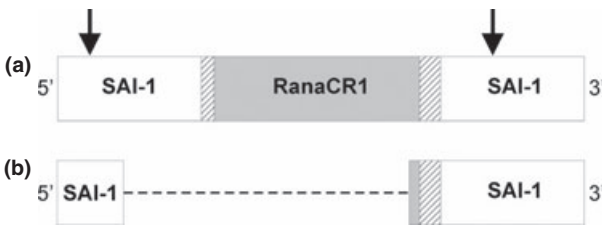

Fig. 1 Diagram of serum albumin intron-1 of (a) *Pelophylax ridibundus* and (b) *P. lessonae* (after Plötner *et al.* 2009). SAI-1 = serum albumin intron-1; *Rana*CR1 = non-LTR retrotransposon of the chicken repeat (CR) family; 5′ dashed box contains target site duplication (TSD) and inverted repeat unit; 3′ dashed box represents 3′UTR with CA repeat, octameric repeat, TSD, and inverted repeat; arrows indicate primer binding sites of Pel-SA-F1 (left) and Pel-SA-R2.

Table 1 Expected sizes of PCR products including primer sequences of different species of *Pelophylax* using the newly developed primer pair PEL-SA-F1 and PEL-SA-R2

Species	Genbank accession number	PCR Product (bp)
P. ridibundus	FN432363–5, FN432371	839–843
P. kurtmuelleri	FN432366–67	717
P. bedriagae	FN432368	841
P. cf. *bedriagae*	FN432372–73	839–840
P. epeiroticus	FN432369–70	838
P. cretensis	FN432376	847
P. cretensis	FN432374–75	828
P. perezi	FN432377	853
P. saharicus	FN432379–80	845–849
P. nigromaculatus	FN432386	379
P. lessonae	FN432383–85	306
P. lessonae (*P. bergeri*)	FN432368	298

Initial PCRs were performed on fifteen individuals: three *P. ridibundus*, three *P. lessonae* and nine *P. esculentus* (three of each karyotype) that had been diagnosed with other methods (see below) in a total volume of 10 μL, each containing 2.0 μL 5× buffer (Promega Go-Taq), 0.24 μM of each primer, 200 μM dNTPs, 0.4 units GoTaq and 0.8 μL of DNA (20–80 ng/μL). After testing various annealing temperatures, we chose the highest temperature that would reliably yield PCR products: the thermocycling profile comprised an initial denaturation at 94 °C for 90 s, followed by 35 cycles of denaturation (30 s at 94 °C), annealing (40 s at 59 °C) and elongation (100 s at 72 °C), and a final elongation step at 72 °C for 10 min. Three microliters of product were run on a 1.5% agarose gel (Fig. 2). While amplicons of *P. ridibundus* yielded one or two bands of approximately 800 bp, there was always only a single band in *P. lessonae* of <350 bp. In the hybrid individuals, we observed both the large and the small band and a faint band of approximately 600 bp. To verify the identity of the products, we initially prepared two amplicons per taxon for sequencing. Later throughout the project, eleven additional individuals were sequenced (Table S1, Supporting information). All sequences were submitted to Genbank (JQ965502–JQ965529). Single band amplicons were cleaned with the ExoTSAP procedure for which 0.0225 units of TSAP (Promega) and 1.2 units of Exonuclease I (New England Biolabs) were combined with 1.5 μL PCR product, incubated at 37 °C for 15 min followed by a heat inactivation step at 80 °C for another 15 min. For amplicons of *P. esculentus*, all three bands were cut out and cleaned with the QIAquick Gel Extraction Kit (Qiagen). All fragments were cycle sequenced in both directions with the same primers as mentioned earlier using BigDye chemistry (Applied Biosystems). Sequencing was carried out on

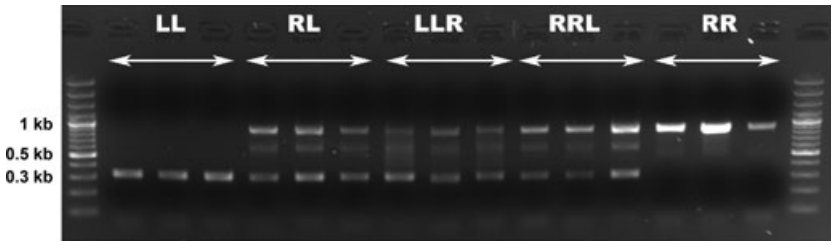

Fig. 2 Gel image showing the PCR products of SAI-1 of 3 individuals each of *Pelophylax lessonae* (LL), diploid *P. esculentus*, triploid *P. esculentus* (LLR and RRL) and *P. ridibundus* (RR). In *P. lessonae* one band of approximately 0.3 kb is visible and in *P. ridibundus* one (or 2) bands (second individual) of approximately 0.85 kb. In the hybrids, both large and small band are seen, as well as a faint hybrid band. Three μl PCR product were run out on a 1.5% agarose gel.

an ABI 3130XL automated DNA sequencer (Applied Biosystems). Chromatographs were checked and edited using CodonCode Aligner (v. 2.0.6; Codon Code Corporation) and compared with Genbank entries.

After confirming that the primers amplified the expected region of serum albumin intron-1, we wanted to determine whether this approach would also provide a means for discriminating the three different karyotypes of *P. esculentus*, RL, LLR and RRL. We therefore used the forward primer fluorescently labelled with a 6-FAM tag and conducted the PCR as before. After checking the PCR product on agarose gel, we combined 1 μL of the diluted PCR product with 10 μL of a 0.83% v/v mixture of the size standard LIZ-600 in HiDye Formamide (both Applied Biosystems). We used this size standard despite the fact that it does not correctly size fragments <580 bp, because we were not interested in the exact length of each fragment but in the intensity of each peak (peak height in relative fluorescence units, RFUs). For every individual, the fragment size(s) and intensities were analysed using GENEMAPPER software (Applied Biosystems) and the peak height ratio (PHR) calculated as the ratio of the R to the L peak was determined. To evaluate the reproducibility of PHRs, we repeated the PCR for five individuals (2 LLR, 2 RRL and 1 RL), ran each sample ten times and calculated the statistical error.

We screened a total of 160 *Pelophylax* (34 *P. lessonae*, 31 *P. ridibundus* and 95 *P. esculentus*) from Poland ($N = 73$), Sweden ($N = 53$), Lithuania ($N = 16$) and Latvia ($N = 18$) that had been diagnosed with other methods to species and karyotype (Table S1, Supporting information). Phenotypes of all specimens had been assessed by morphology (Berger 1966; Plötner 2005, 2010). In all Polish samples, ploidy levels had been confirmed by measurements of erythrocyte size (Ogielska *et al.* 2004; Kierzkowski *et al.* 2011), and L and R chromosome sets were distinguished by using the AMD/-DAPI method (Heppich *et al.* 1982 and Ogielska *et al.* 2004). In 50 of these samples, karyotypes had been assessed additionally by electrophoresis of LDH-B, an allozyme marker diagnostic for the three taxa (Uzzell & Berger 1975; Hotz *et al.* 2008). For this, a slightly modified protocol was used: tissues were homogenized in ice-cold buffer consisting of 20 mM TRIS, 1 mM EDTA, 0.5 M sucrose pH 7.5, at 4 °C. The supernatants were used in PAGE electrophoresis, which was carried out at pH 8.6 using TRIS/glycine buffer according to Hofman *et al.* (2012). Thirty-eight of the Polish samples and all 53 samples from Lithuania and Latvia had been genotyped for 18 microsatellite loci, while the 53 samples from Sweden had been genotyped with nine diagnostic microsatellite markers (see Tables S2a,b, Supporting information for details on the microsatellite genotyping). The results of the Lithuanian and Latvian samples had been published in Christiansen & Reyer (2011).

In addition to the 160 samples that had been identified with other methods, we also tested this new approach on 19 buccal swab samples collected during a survey of water frogs from northern Germany (Table S3, Supporting information). For these, we did not verify the karyotype but only compared the banding pattern on an agarose gel with morphological identification to determine whether DNA derived from buccal swabs would also work for this application.

Results

We obtained PCR products of different sizes for each species and a combination of products for the hybrid that allow for easy discrimination via agarose gel electrophoresis (Fig. 2). While in *Pelophylax lessonae*, the PCR always resulted in a single small band that on the agarose gel could be estimated to be approximately 300 bp in length, the bands seen in *P. ridibundus* were never of this size (Table 2). The most common band in this taxon was approximately 850 bp in length. Three RR yielded an additional smaller band of approximately 700 bp, and one individual only showed the smaller fragment. One individual of RRL karyotype was also heterozygous for the two *ridibundus* alleles. In the hybrids, both the small and the larger bands were visible. In addition, most of

Table 2 Number of individuals screened and percentage of individuals correctly assigned to taxon and karyotype. Peak heights were measured in relative fluorescence units (RFUs) and the peak height ratio is the ratio of the large (*ridibundus* specific) to the small (*lessonae* specific) peak

Karyotype	LL *N* = 34	RL *N* = 34	LLR *N* = 29	RRL *N* = 32	RR *N* = 31
Correct taxon diagnosis based on PCR banding pattern (agarose gel)	Band < 350 bp 100%	Band < 350 bp + band(s) > 650 bp 100%			Band(s) > 650 bp 100%
Correct karyotype diagnosis based on range of peak height ratio (PHR) (automated sequencer)	0 100%	0.16–0.32 94%	<0.17 93%	0.33–0.7 91%	>3.0 100%

these amplicons also showed a third faint band of approximately 600 bp in length. This band was identified as a hybrid band that was only seen when PCR products were not denatured (as on agarose gels), but not when denatured products were separated on an automated sequencer. With the DNA extracted from the buccal swabs, the same type of banding pattern was generated, and there was no difference in intensity of the bands compared with the ones generated from tissue-derived DNA. Banding pattern agreed with morphological identification in most cases and provided a means of identification for those animals in which morphological assignment had been uncertain (Table S3, Supporting information).

The 258-bp sequences (305 bp including primers) derived from LL animals as well as from the smallest PCR bands generated from samples of *P. esculentus* were all identical and matched Genbank entries FN432384 and 432385 sampled from Germany, as well as FN42383 from northern Italy, and are from now on referred to as L allele. The agarose banding pattern of all 34 LL screened showed only a single band of the expected sized fragment. The sequences obtained from the large band of *P. ridibundus* and of *P. esculentus* (R allele) were 795 bp long (842 bp including primers). They were all identical and matched with FN432365 derived from a Polish *P. ridibundus*. The smaller band found in four individuals was sequenced from three individuals and yielded a sequence of 669 bp length (716 bp including primers) that matched FN432366 and FN432367, derived from *P. kurtmuelleri* (Greece) except for a single nucleotide substitution.

The fluorescently labelled PCR products were sized as 303 bp for the L allele (Fig. 3a), 772–782 bp for the large R allele (Fig. 3e) and 696–699 bp for the small R allele. While in all LL, we only detected a single peak as also seen on the agarose gel, almost half (*N* = 15) of all the RR samples also yielded an additional very small peak of the size of the L allele, which was never detected on the agarose gel. This small peak was reproducible among runs and different PCRs. In *P. ridibundus*, the PHR, the ratio of RFUs of the R to the L, ranged from 3.08 to 88.54 (avg. 30.26 ± SE 6.9). In the hybrids, we always detected the L and one or two R alleles, and PHRs differed to some extent between karyotypes (Fig. 3b–d). PHRs were reproducible between runs, PCR reactions and differently diluted samples of the same PCR product, but only if the peak intensity of the strongest peak was <8000 RFUs. Therefore, all samples yielding peaks of higher intensity were diluted accordingly. Among the five samples for which the PCR product was run ten times on the automated sequencer to evaluate the reproducibility of this method, the average standard error of the PHRs was 0.0065 (0.0020–0.0157). Among the PHRs derived from 95 *P. esculentus* samples, three groups could be recognized that would optimize correct assignment: (i) PHRs of 0–0.17 were considered diagnostic for LLR karyotypes, (ii) PHRs of 0.17–0.32 contained mainly RL individuals and (iii) values >0.32 were diagnostic for RRL individuals (Fig. 4, Table S1, Supporting information). According to these criteria, we were able to correctly assign 93% of the hybrid individuals to their karyotype (Table 2). While for diploid hybrids, the success rate was 94%, for LLR it was 93%, and for RRL karyotypes correct assignment was only achieved in 91% of the cases.

Discussion

We have developed a fast and cost-effective method to reliably identify species of the *Pelophylax esculentus* complex. A single small band of 300 bp is diagnostic of *P. lessonae*, one or two bands that are >700 bp are diagnostic of *P. ridibundus* and a combination of large and small bands indicates *P. esculentus*. Assignment to the taxon level based on agarose gel screening of a PCR product was identical to that derived by one other or a combination of more elaborate methods. Our approach was developed on the basis of the characterization of SAI-1 (Plötner *et al.* 2009), and it seems to exceed a recently proposed method by Patrelle *et al.* (2011) in reliability, in addition to being cheaper and faster. All sequences of alleles of *P. ridibundus* and *P. lessonae* were identical with those generated

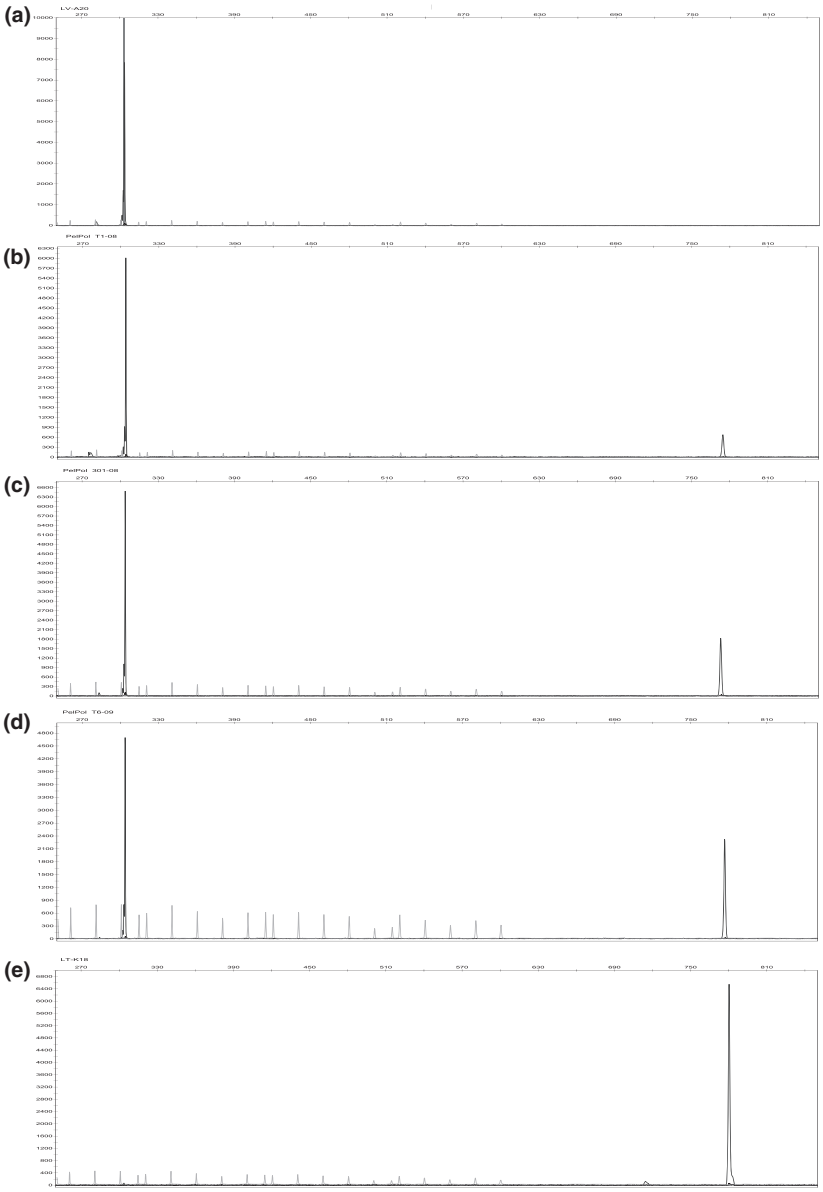

Fig. 3 Genotyper graphs showing representative sizes and peak heights for (a) *Pelophylax lessonae* (LL); (b) *P. esculentus*, *triploid* (LLR); (c) *diploid* (LR); (d) *triploid* (RRL); (e) *P. ridibundus* (RR).

by Plötner *et al.* (2009). Interestingly, among our samples, we found the *P. kurtmuelleri* allele in four individuals (three from Poland and one from Latvia. In the samples from Poland, there was nothing unusual regarding any of the other morphological and molecular markers that had been analysed. The individual from Latvia was heterozygous with one common and one private allele in two of the loci (Rrid064A: 211, and Re1CAGA10: 159). It will be interesting to determine whether this allele has been maintained in *P. ridibundus* (and *P. esculentus*) at low frequency throughout the range or if there has been introgression of the Balkan taxon into the north.

Our method was highly accurate (100%) for taxon identification of the Central European water frogs and even worked well on buccal swab samples, but it was not as successful for determination of the karyotype. The distinction among *P. esculentus* karyotypes RL, LLR and RRL with fluorescently labelled primers on a sequencer using the PHR approach showed a lower reliability of only 93%. Seven individuals were assigned to another hybrid category than had been determined by other methods. Interestingly, two of the individuals (one RRL, No. 76, and one LLR, No. 91) that fell into the PHR range of RL had also a pattern of bands of LDH-B that had never been found before; this pattern is currently being described (Dziewulska-Szwajkowska and Ogielska, work in progress). Therefore, these individuals may have been recombinants. Evidence of triploid water frogs possess-

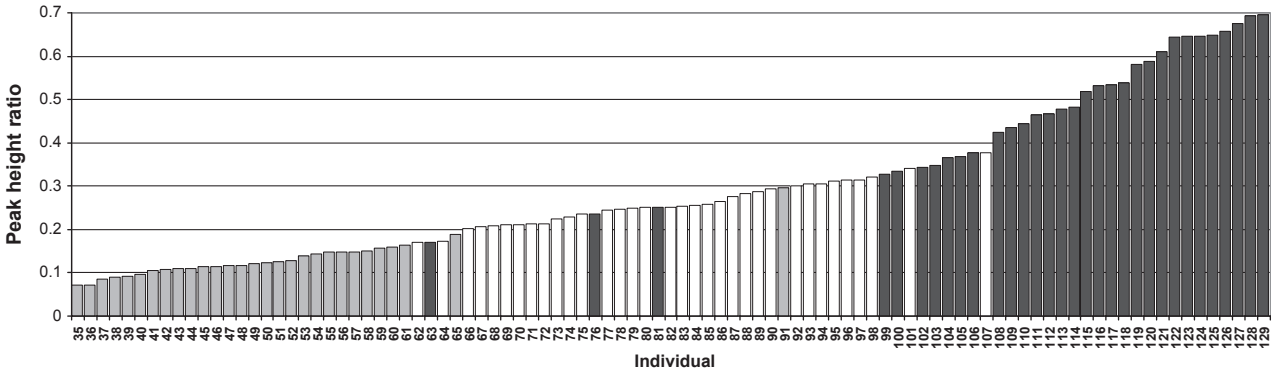

Fig. 4 Diagram showing peak height ratio of 95 *Pelophylax esculentus* of three different karyotypes: LLR (grey bars, $N = 29$), RL (white, $N = 34$), RRL (black bars, $N = 32$). Individuals are numbered as in Table S1.

ing two L and one R allele at some loci while other loci possess two R and one L allele was formerly provided by Uzzell & Berger (1975), Günther *et al.* (1979), and Hotz *et al.* (2008). However, two other individuals (No. 36 and 50) showing the very same, previously unknown, pattern of LDH-B, were assigned correctly. It will be interesting to compare the success rate of our method in populations with different rates of introgression and recombination. We currently have no explanation for the occurrence of the low intensity L peak in some RR samples. It cannot be ruled out that this was caused by contamination in the laboratory. Regardless of the origin, the intensity of the L allele in the RR samples was always so low that it never made identification questionable.

Although it would be most desirable to find a PCR-based technique that can distinguish both taxon and karyotypes easily and error-free, this goal seems to have inherent difficulties. Karyotype determination works better, if the length difference between the alleles examined is small. This is because a longer allele generally amplifies poorer than a shorter allele (e.g. Fig. 3 and Table S1). Microsatellite alleles with just a few base pairs difference are thus highly reliable for ploidy determination (Christiansen & Reyer 2011). With the large length difference of the L- and R-specific band of SAI-1, strong amplification bias towards the shorter allele squeezes the PHRs for all karyotypes together. This results in gradual transitions of PHRs between the karyotypes that make it difficult to clearly define karyotype-specific ranges of PHR. Considering a general amplification bias towards the smaller allele, one would expect that most misidentifications would be due to this phenomenon, that is, more RRL individuals incorrectly identified as RL and RL individuals identified as LLR. Interestingly, we did not find evidence for such a trend: of the seven individuals that had been misidentified, four had L bands of lower intensity compared with the large band than expected for the correct karyotype category (two LLR identified as RL and two RL identified as RRL).

The large length difference between the alleles examined does on the other hand provide high L and R genome specificity. It is highly unlikely that one allele could mutate to the length of the other. With microsatellite alleles often having just a few repeats difference in between, such size-homoplasy apparently arises relatively often in geographic areas with high allele diversity (Christiansen & Reyer 2011). Therefore, a reliable method for distinguishing both taxon and karyotype may consist of a combination of markers with large and small allele differences, as for example the present method with genotyping a few microsatellites.

Our method should allow distinction between other taxa with similar ease, given that the allele sizes found so far (Table 1) are representative for each taxon. While unfortunately this method does not discriminate in size between alleles derived from *P. bedriagae*, *P.* cf. *bedriagae* and *P. ridibundus*, it now should be possible to identify parental and hybrid taxa of two other hybridogenetic systems. *P. grafi* is the hybridogenetic taxon deriving from crosses between *P. perezi* and *P. ridibundus* or *P. esculentus* (Graf *et al.* 1977; Graf & Polls Pelaz 1989; Pagano *et al.* 2001) that occurs in southern France and north eastern Spain. A size difference of approximately 10 bp is expected between *P. perezi* and *P. ridibundus*, which may require the use of a polyacrylamide gel to separate the bands as it has higher resolving power than agarose. Resolution on agarose gel should be sufficient to distinguish PCR fragments of individuals of the third hybridogenetic complex: *P. hispanicus*, the Italian hybrid frog, is the result of hybridization of *P. bergeri* with *P. ridibundus*, and the expected size difference between alleles is 545 bp. To distinguish parental and hybrid taxa of both complexes, allozyme markers have been used until now (Günther & Plötner

1994; Hotz *et al.* 1994). The identification of other taxa or hybrids, for example, the recently discovered hybrids between *P. perezi* and *P. lessonae* (Pagano *et al.* 2001) should also be straightforward.

Our method provides a simple and cost-effective means to distinguish among members of the *P. esculentus* complex that works well also on noninvasively collected samples. This will aid in elucidating the distribution of *P. lessonae*, a taxon that is particularly threatened in Central Europe, as well as in the identification of alien taxa (and their hybrids). We are at present successfully applying this technique on request of planning agencies conducting environmental impact statements.

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J.S.H. developed the method and analyzed the data. M.H. screened many samples with the new method. M.O. provided selected material from her collection and coordinated, supervised and interpreted LDH-B and microsatellite analyses in Poland. D.G.C collected the samples from Sweden, Latvia and Lithuania and genotyped them with microsatellites. D.D.-S conducted the LDH-B analyses, and E.C. the microsatellite analyses of the Polish samples. J.S.H., M.V., D.G.C., and M.O. wrote the manuscript.

Data accessibility

DNA Sequences: Genbank accessions JQ965502–JQ965529.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Sample information and data.

Tables S2a Multiplex composition, specificity of locus and original reference for each locus.

Tables S2b Cycling parameters for multiplex reactions.

Table S3 Sample information of swab samples.

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