DISCRIMINATION OF MOOR FROG (*RANA ARVALIS*) AND COMMON FROG (*RANA TEMPORARIA*) INDIVIDUALS USING A RAPD TECHNIQUE

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A method has been developed for discriminating between the common frog (*Rana temporaria*) and the moor frog (*Rana arvalis*) using either of two primers in RAPD analysis of DNA samples extracted from larval tail tips. These two frog species can be extremely difficult to distinguish morphologically at the egg clump and larval stages, which are very convenient stages for monitoring populations when there are conservation concerns. The adults need capture and detailed morphological examination to effect certain identification, this being particularly true for edge-of-range populations. The two primers also distinguished DNA samples of common toad (*Bufo bufo*), natterjack toad (*Bufo calamita*), pool frog (*Rana lessonae*) and the marsh frog (*Rana ridibunda*). Additionally, findings are reported for a third primer which distinguished, intraspecifically, between relatively closely located common frog (*Rana temporaria*) populations in southern England.

Key words: anura, frog identification, molecular genetics, population assessment

INTRODUCTION

Palaearctic frogs of the genus *Rana* have been categorised into two groups: the "water" (or "green") frogs and brown (or "grass") frogs. Water frogs are predominantly aquatic, often green in colour, and are typically found in freshwater shallows, or basking near the waterside. Brown frogs are, conversely, predominantly terrestrial, normally brown coloured, and spend most of their time concealed in herbage (hence "grass" frogs), often at considerable distances from open water. The north-west European water frogs are the marsh frog *Rana ridibunda,* the pool frog *Rana lessonae* and the edible frog *Rana esculenta*, while the brown frogs in the same region are the common frog *Rana temporaria,* the moor frog *Rana arvalis* and the agile frog *Rana dalmatina* (Matz & Weber, 1983; Nöllert & Nöllert, 1992; Arnold, 1995; Gasc, *et al.* 1997; Arnold & Ovenden, 2002).

Accurate species identification is essential in ecological studies and in population monitoring programmes responding to concerns about global amphibian decline. The European ranges of *Rana temporaria* and *Rana arvalis* overlap substantially, and adult individuals of the two species are not always easy to distinguish morphologically (Fig. 1). While useful morphological indicators such as the size and shape of the metatarsal tubercle on the inner rear toe are available (but only after capture) for adults, species identification in earlier developmental stages – which can be particularly valuable in population monitoring – is much more problematic. One effective brown frog population census method is the counting of spawn clumps (Griffiths & Raper, 1994; Loman, 1996). In brown frog census work in southern Sweden, Loman (2001) found that up to 15 percent of spawn clumps belonging to either *Rana temporaria* or *Rana arvalis* could not be discriminated on morphological grounds. Again,

larvae of the two species are difficult to distinguish, the identification problems being compounded by the phenotypic plasticity of anuran larvae (Vences *et al.*, 2002). Indeed, recent claims for new *Rana* species in the Pyrenees based on substantial geographical variation shown by apparent *R. temporaria* populations (Vences, 1992; Arano *et al.,* 1993; Vences *et al.*, 1998; Veith *et al.*, 2002) may just reflect morphological variability in *R. temporaria* tadpoles (Vences *et al*., 2002). However, it may indeed be the case that unrecognised and new species occur in some European brown frog populations. These considerations prompted us to develop an unambiguous molecular method for identifying *R. temporaria* and *R. arvalis* individuals, applicable to developing eggs, larvae and adults.

The methodology we selected was the DNA-based RAPD technique (Williams *et al.*, 1990, 1993), which has proved useful in genetic studies of rare and endangered amphibian populations (Kimberling *et al*, 1996),

FIG. 1. An illustration of identification difficulties that can present with the two brown frogs *Rana temporaria* (left) and *Rana arvalis* (right). Handbook descriptions usually describe the moor frog as normally being stripe-backed and with a more pointed snout than the common frog. In this illustration the randomly caught common frog (from a London garden) on the left has a bolder stripe and a more pointed snout than the moor frog (from southern Sweden) on the right.

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and which we have recently used to clarify the genetic affinities of different populations of the pool frog *R. lessonae* (Snell *et al.*, 2005). More pertinently, RAPD has been shown to be effective in discriminating amphibia at the species level in the cases of green frogs of the species *R. esculenta*, *R. lessonae* and *R. ridibunda* (Zeisset & Beebee, 1998), and larval toads of the species *Bufo bufo* and *B. calamita* (Bardsley, *et al.*, 1998).

We report here a RAPD method in which either of two primers clearly and correctly identified individuals from a large sample group as either *Rana temporaria* or *Rana arvalis*; the two primers also gave distinct, species-specific band patterns for *R. esculenta*, *R. lessonae*, *B. bufo* and, in the case of primer OT-A3, *B. calamita*. It had been hoped to include *R. dalmatina* in the study, but no tissue samples were available. RAPD data for a third primer are also reported, because, though less useful in species diagnosis, this primer discriminated intra-specifically, between different southern English populations of *R. temporaria*.

MATERIALS AND METHODS

TISSUE SAMPLES

Egg samplings (ca. 30 ova from each site) from *Rana arvalis* were obtained from frogs from southern Sweden, Denmark and Poland. The adults were clearly identified by their metatarsal tubercles and the spawning was at least ten days later than is normal for *Rana temporaria*. *Rana temporaria* eggs were collected in late February from ponds in S.E. London, Bromley, Bexley, Suffolk and Dorset (all UK sites). In order to produce tissue containing enough DNA for extraction and recovery, the eggs were placed in separate tanks (labelled according to species and population), where they were allowed to develop. The water used was filtered, conditioned ("ReptiSafe" treated – see below) mains tapwater, which was allowed to stand for 10 days, then seeded generously with *Daphnia* as natural water-filtering agents. When the larvae had reached ca. 15-20 mm in length, small sections of tail fin tip (stored in absolute alcohol after removal) were used as a source of DNA and the larvae returned to the tanks to allow the tail tips to begin to part-regenerate naturally. The larvae were finally released back into their ponds of origin. The loss of tail tips in anuran larvae has been shown to cause little loss of ecological fitness and may in fact be a mechanism to reduce predation (Wilbur & Semlitsch, 1990; Vences *et al.*, 2002) somewhat analogous to tail loss in lizards.

SAMPLE SIZES

Primer OT-A3: *Rana arvalis*: Denmark, 26; Sweden, 21; Poland, 15. *Rana temporaria*: Suffolk, 24; Bexley, 19; Bromley, 17; S.E. London, 18; Dorset 17.

Primer CS-L1: *Rana arvalis*: Denmark, 21; Sweden, 24; Poland, 24. *Rana temporaria*: Suffolk, 26; Bexley, 17; Bromley, 16; S.E. London, 18; Dorset 17.

Primer OT-C6: *Rana arvalis*: Denmark, 12; Sweden, 14; Poland, 14. Rana temporaria: Suffolk, 28; Bexley, 13; Bromley, 17; S.E. London, 17; Dorset, 15. *R. arvalis* numbers were substantially lower for primer OT-C6 than those used for primer OT-A3 & CS-L1 as, with this primer, the main focus was on *Rana temporaria*.

REAGENTS

Agarose, deoxyribonucleotides and 100 base-pair DNA marker ladders were obtained from Gibco-BRL, UK. Chelex 100 resin was from Bio-Rad (CA., USA). DNA polymerase derived from the organism *Thermus islandicus* ("Thermoprime Plus ") was obtained from Advanced Biotechnologies, Epsom, UK. PCR buffers were supplied with the enzymes. Primers were synthesized by Operon Technologies (Gosforth, UK.), Cruachem (Glasgow), and Microzone (Lewes, E. Sussex). All other chemicals were from Sigma Chemical Co., Poole, Dorset, UK, and solutions were made using sterile distilled water. Aquarium water conditioning agent was "ReptiSafe" (Zoo Med Inc., CA. or, in the UK, from Livefood UK online).

DNA EXTRACTION

Tissue fragments (c. 4 mg) were statically incubated in 160 µl of sterile distilled water and 40 µl Chelex-100 resin overnight in a water bath at 55°C (Walsh *et al.*,1991; Zeisset & Beebee, 1998). The samples were then briefly vortexed, boiled for eight minutes in a water-bath, re-vortexed and centrifuged at $5000 \times g$ for three minutes at room temperature. The resulting supernatant (stored at –20°) was used as the DNA source for subsequent PCR amplifications. DNA concentrations were determined by measuring absorbance at 260 nm, and were adjusted to approximately 50 µg/ml by dilution or freeze-drying (100 µg/ml and 25 µg/ml gave the same PCR amplification results as 50 µg/ml). All procedures were carried out in designated pre- and post-PCR areas. All solutions and apparatus were also confined to pre- or post-PCR areas and rigorously sterilized where appropriate. Freshly autoclaved pipette tips and PCR-dedicated, thin-walled microfuge tubes (Advanced Biotechnologies, Epsom, Surrey, UK.) were used throughout.

RAPD ANALYSIS

RAPD-PCR was essentially that described by Williams *et al.* (1993) with some modifications. Each PCR assay contained 2 µl of extracted supernatant (with DNA) in a final volume of 20 µl with 100 µM each of dATP, dCTP, dGTP, dTTP, 0.2 µM 10-mer oligonucleotide primer and 0.8 units of DNA polymerase. Addition of the enzyme supplier's buffer to each reaction resulted in final reaction concentrations of 75 mM Tris- HCl (pH 8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$ and 0.01% (v/v) Tween 20. Glycerol was added to a final concentration of 5% (v/v).

TABLE 1. Primer OT-A3 band patterns in other anuran species. Sizes in average base pair numbers. Heavy bands shown in bold. Provenance of samples: *Bufo bufo*, larvae from southeast London, *Bufo calamita*, larvae from captive colony, original provenance Norfolk, UK, *Rana ridibunda*, larvae from North Kent Marshes and *Rana lessonae* larvae from a captive colony of mixed origin.

Species	Sample no.	Band sizes (bp): heavy bands are shown in bold.											
Bufo bufo	4			719	678					522	**499**		
Bufo calamita	3	**948**	839	719				572					
Rana ridibunda	4					**653**			**560**		500		428	**341**
Rana lessonae	6			722			**643**	575			497	**438**	350

Using either of two thermocyclers (Techne PHC3 and a Techne "Genius"), a thermocycling protocol was used which started with a denaturation cycle of 94°C for four minutes followed by 40 cycles of three segments (with a ramp rate of 60%) consisting of: 94°C x 1 minute, 36°C x 1 minute, and 72°C x 2 minutes, with a final extension cycle of 72°C for six minutes. On completion of the reaction, 5 µl of loading buffer (containing 2.5 mg/ml bromophenol blue) were added and the mixture electrophoresed at 4 V/cm through 1.5 % w/v agarose in TBE (67.5 mM Tris-borate, 1.5 mM EDTA (pH 8.0)) running buffer, allowed to run for 5 to 6 cm, then stained in a bath of sterile distilled water containing 1mg/l ethidium bromide .

Typically, three wells of each gel were loaded with DNA molecular weight standards, and one well contained, as a control, the products of a PCR reaction using all of the reagents except DNA. Gel images were captured by a video camera linked to a computer and digitised (GDS-7600 Gel Documentation System, UVP Ltd., Cambridge, UK).

RESULTS

PRIMER SELECTION

One hundred and five 10-mer primers were tested in preliminary experiments (not reported here) and two of these, OT-A3 and CS-L1, were chosen as being the most discriminating between *Rana temporaria* and *Rana arvalis* DNA on the basis of polymorphic band resolution and repeatability: these were used in later analyses. A third primer, OT-C6, which separated the two species less effectively, but seemed capable of discriminating quite closely situated *R. temporaria* populations, was also chosen for further work. To test reproducibility, replicate experiments, seven for each of the three primers, were made. Each successive gel run used an increasing number of samples, which included DNA from the individuals used in the previous run plus DNA from new individuals and populations: all gave the same results except in the case of primer 3 where it became increasingly obvious that different *Rana temporaria* populations were producing varied banding patterns (see below). The results from the two different thermocyclers were entirely comparable.

RESULTS FOR SELECTED PRIMERS

Primer OT-A3 (AGTCAGCCAC). (NB Primer sequences all given 5' to 3'). This primer gave band patterns that were clearly different for *Rana temporaria* (95 individuals tested) and *Rana arvalis* (62 individuals tested; Fig. 2). Two bands were highly diagnostic for *R. temporaria* (407 bp band in 97% of individuals tested, 910 bp band in 90%) and one band was completely diagnostic for *R. arvalis* (560 bp band in 100%). As a further check on the species specificity and utility of this primer, it was tested with DNA samples from four other NW European anuran species (Table 1): the band patterns were identical for all individuals of a species (*R. lessonae*, *R. ridibunda*, *Bufo bufo* and *B. calamita*), and were species specific, differing clearly from the patterns for *R. temporaria* and *R. arvalis* (Fig. 2)

Primer CS-L1 (TCCCTTCCTC). This also yielded highly distinctive band patterns for *R. temporaria* (94 individuals tested) and *R. arvalis* (69 individuals tested; Fig. 3). Again, for *R. arvalis*, one, possibly complex, band (380 bp) occurred in all the *R. arvalis* individuals, and in none of the *R. temporaria* individuals tested with this primer. Also, 68% of *R. temporaria* individuals gave a strong band at 540 bp, with no matching band in *R. arvalis*. This primer was tested for species specificity with DNA samples from three other NW European anuran species (Table 2), and again, the band patterns were uniform within species, and species-specific.

Primer OT-C6 (GAACGGACTC). This primer was interspecifically discriminating, though less so than primers OT-A3 and CS-L1, but it showed an interesting ability to distinguish four different southern English populations of *Rana temporaria* (Fig. 4). Compared to the rural populations in Dorset and Suffolk, the urban S.E. London population lacked a heavy band at about the 445 bp position (see Fig. 4, arrow 2). The more suburban populations of Bromley and Bexley lacked a band at about the 680 bp position (Fig. 4, arrow 1) when compared with the Dorset and Suffolk populations. Larvae of *Rana temporaria* separated by only around 5 km / 3 miles (Bexley or Bromley to the S.E. London collection site) were distinct in two regions (the two positions indicated by arrows 1 and 2 in Fig. 4).

DISCUSSION

In this study we have described a RAPD method which, using either of two primers, unambiguously identified larvae of varied geographical origin as either *Rana temporaria* or *Rana arvalis*. Reproducibility is sometimes a concern in RAPD experiments, however, high reproducibility of band patterns was seen in the replicate amplifications carried out here, and it can also
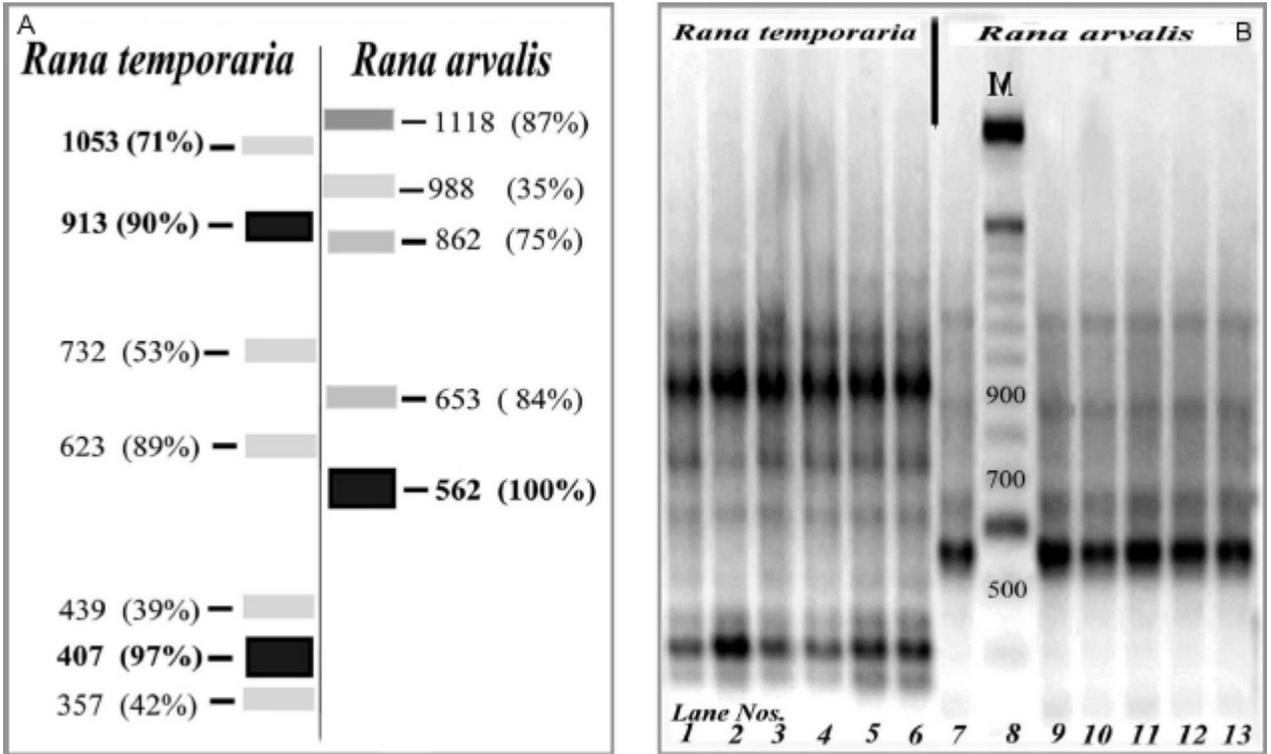
FIG. 2. RAPD results for *Rana temporaria* and *Rana arvalis* using primer OT-A3. A, graphical synopsis of primer OT-A3 results based on 157 lanes (=individuals). It is evident that many bands are inter-specifically discriminating. Rectangles shown in bold represent bands which gave strong amplification results as well as being interspecifically discriminating. B, part of one of the primer OT-A3 gels, representative of this primer's results overall, and demonstrating the clarity of species separation. Lanes 1-6, *R. temporaria*; lanes 7 and 9-13, *R. arvalis*; lane 8, molecular weight markers (100 bp DNA ladder).
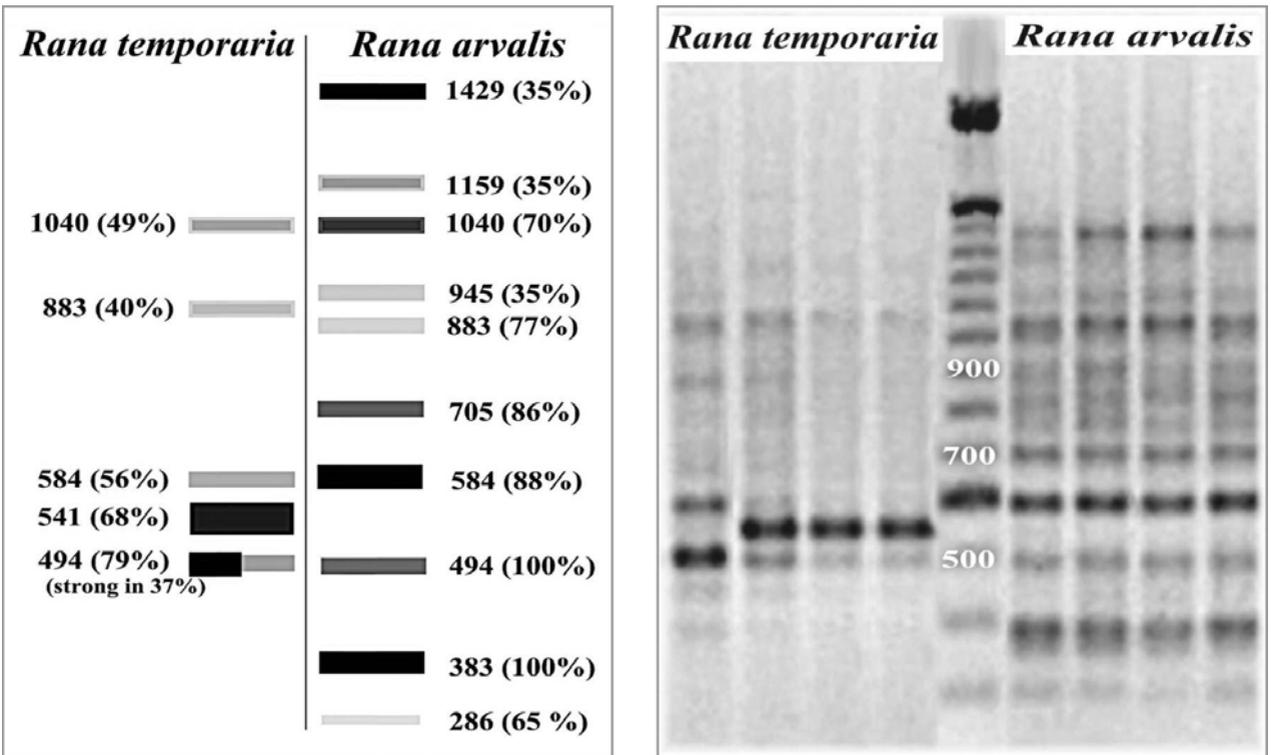

FIG. 3. RAPD results for *R. temporaria* and *R. arvalis* using primer CS-L1. A, Graphical synopsis of primer CS-L1 results based on 163 lanes (= individuals). Initial numbers are average band lengths in base pairs. Parenthesised numbers represent percentage of that species with that band. Rectangles shown in bold represent bands which gave strong amplification results and contributed to the species specificity of the band patterns. A, Part of one of the primer CS-L1 gels, representative of this primer's results overall, and indicating the distinctiveness of the band patterns for the two species. Lanes 1- 4, *R. temporaria*; lanes 6-9, *R. arvalis*; lane 5, molecular weight markers (100 bp DNA ladder).

TABLE 2. Primer CS-L1 band patterns in other anuran species. Sizes in average base pair numbers. Heavy bands shown in bold. Provenance of samples: *Bufo bufo*, larvae from S.E. London, *Rana ridibunda*, larvae from north Kent marshes and *Rana lessonae* larvae from captive colony of mixed origin.

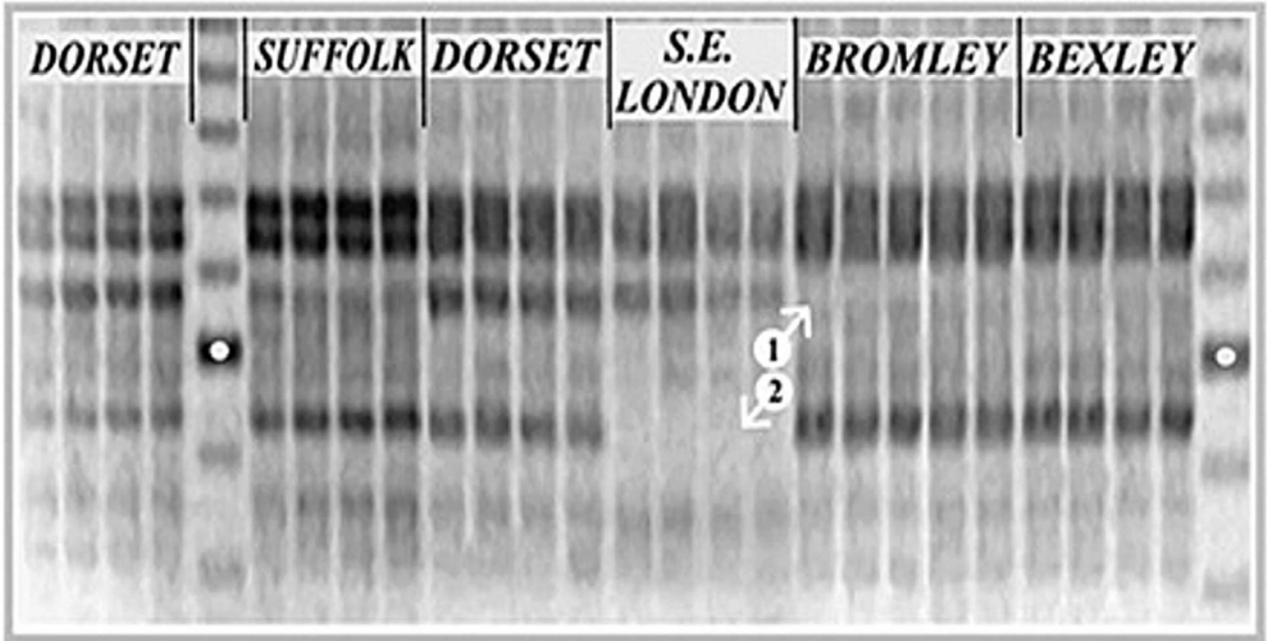| Species	Sample no.	Band sizes (bp). Strong bands shown bold						
Bufo bufo	4	827	661		560	**481**	**424** 352	
Rana ridibunda	3		738	**654**		495		303
Rana lessonae	8		693		**522**	499		

FIG. 4. A section of a primer OT-C6 gel where all samples were *Rana temporaria*. The gel contains two internal 100 base pair marker lanes (the fifth and last lanes), the 600 base pair position of which has been marked with a white spot. The two main points of dissimilarity are marked with directional circles numbered 1 and 2.

be pointed out that results reported in our recent pool frog study (Snell *et al.*, 2005) have been replicated by different workers in the Greenwich laboratory, in an extension of that investigation.

Primer OT-A3 gave a particularly clear contrast in banding patterns for the two species, with a 560 bp band being a strong, unique marker for *R. arvalis* (Fig. 2), whereas with primer CS-L1 the *R. arvalis* patterns were more complex (see Fig. 3), though there was a distinct and unique *R. arvalis* band at 380 bp. It is also helpful that the primers discriminated individuals of other anuran species (see Tables 1 and 2). The technique is relatively inexpensive and not too time-consuming: DNA extraction needs up to one hour's manipulation followed by incubation overnight. Using fast thermocyclers the work can then be completed within a working day, furthermore, a single large format electrophoresis tank running several rows of wells on large gels can analyse upwards of 120 samples. Tail tips can be collected quickly from large numbers of larvae with low risk of larval mortality, even for very small tadpoles (Gosner stage 26 and above: Gosner, 1960).

We anticipate that our identification method will be most useful in population monitoring in relation to con-

servation concerns using samples from egg clumps in the later stages of egg development and from egg samplings allowed to develop in "captive" conditions (as here) or with wild-caught larvae, however, the method could have wider applications. Adult brown frogs can be very similar in appearance and may need expert guidance to distinguish, especially in the case of northwest European fringe populations of common and moor frogs. Fig. 1 gives an example of this confusing similarity, where a common frog (randomly caught in a London garden) is shown alongside a moor frog (on the right) from a southern Swedish population: the common frog actually resembles most handbook descriptions of the moor frog (with its central dorsal stripe and more pointed snout), and the moor frog is more in agreement with common frog descriptions. Fortunately, and reliably in the case of moor and common frogs, the metatarsal tubercle size can be used. These features are not immediately obvious without close examination and therefore require the capture of animals in order to be useful. Our method could be applied to buccal swabs (Pidancier *et al.*, 2003) or to toe clippings from adults in cases where the metatarsal tubercle size may not have great value (e.g. when there is a possibility of unrecog-

nised sibling or sub-species in the same geographic area). The possible occurrence of cryptic species in European brown frog populations has been previously raised by experienced and well-travelled herpetologists (Bentley, J., Harrison, C., pers. com., 1997). The availability of two species-specific primers could be useful and informative in tests with samples from geographical areas where "cryptic" or sub-species are suspected and the results yield unexpected band patterns. This application may be particularly relevant to the Mediterranean peninsulae where brown frog diversity tends of to be the highest. Even as recently as 1993 a new brown frog species from the Pyrenees area (*Rana pyrenaica*) has been described (Serra-Cobo, 1993).

Interestingly, sub-fossil remains of the moor frog (*Rana arvalis*) and the agile frog (*Rana dalmatina*) have recently and unexpectedly been found in Middle Saxon (c. 600-950 AD) archaeological digs in the fenland districts of England (Gleed-Owen, 1999, 2000)**,** indicating that these species could have been native to Britain, and may have persisted into modern times. Indeed, the close similarity of fringe populations of moor frogs to common frogs could mean that there is a chance of unrecognised remnant populations of the moor frog, especially in more remote areas, which our technique could offer confirmatory identification.

The results reported for primer OT-C6 indicate that even quite closely adjacent populations of *R. temporaria* in southern England are genetically distinguishable, using our RAPD method, though it should be acknowledged that the numbers of individuals sampled are not large, and that codominant markers are more useful for population studies (Brede & Beebee, 2004). In the case of the S.E. London and Bexley/Bromley populations, separated by only around 5 km, our findings are consistent with those of Hitchings and Beebee (1996), which showed that, in urban situations, man-made features such as busy roads can act as efficient barriers to dispersal and migration in common frog populations, and so encourage genetic divergence.

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