HERPETOLOGICAL JOURNAL, Vol. 8, pp. 145-148 (1998)

IDENTIFICATION OF *BUFO* LARVAE BY MOLECULAR METHODS

LOUISE BARDSLEY, SEAN SMITH AND TREVOR J. C. BEEBEE

School of Biology, University of Sussex, Falmer, Brighton BN1 9QG, UK

We have characterized two molecular techniques, protein typing and RAPD analysis, for the identification of two species of European *Bufo* larvae (*Bufo bufo* and *B. calamita*). These tadpoles are very difficult to distinguish on morphological grounds. Protein typing was improved by the use of a sensitive (silver-based) staining method, and replacement of low temperatures with ethanol submergence for tissue storage. Both techniques reliably resolved the two species and required very small amounts (<2 mg) of tissue, which could be obtained easily and without sacrificing the animals.

INTRODUCTION

Amphibians are replete with examples of problematical identification during early developmental stages, and there are many instances where morphological differentiation is impossible or highly unreliable. For many ecological studies, however, accurate identification of amphibian larvae is essential. In Britain, for example, there are two species of *Bufo* (*B. bufo* and *B. calamita*) which sometimes compete during larval development (e.g. Heusser, 1972; Banks & Beebee, 1987), and the tadpoles of these toads are very difficult to distinguish from one another. Both species are uniformly black in colour, though relative sizes, ratios of inter-ocular distance: mouth width, tooth row arrangement and chin patch coloration have been invoked as suitable methods for identification (Smith, 1951; Beebee, 1977; Davis, 1985). All, however, are time-consuming, unreliable, and often damage or kill the subjects of study. Beebee (1990) developed a protein typing method which distinguished spawn jelly proteins, embryos and large tadpoles which (except in the case of embryos) did not require killing the subjects. This approach required relatively large amounts of tissue and was thus impractical for small larvae; furthermore, it required inconvenient cold storage facilities (liquid nitrogen or dry-ice flasks) in the field.

In this study we report on modifications to the protein typing method which render it useful with small larvae and removes the need for cold storage. The development of a simple DNA-based (RAPD) technique (Williams *et al.*, 1990) that achieves the same end is also described. RAPD analysis is useful over a wide range of taxonomic levels, and has been used successfully with amphibians to quantify genetic variation at individual, population and species levels (e.g. Masters & Forester, 1995; Masters, 1995; Kimberling *et al.*, 1996).

MATERIALS AND METHODS

SAMPLING OF TADPOLES

Larvae of both species were sampled at widely different geographic locations within the UK. *B. calamita* and *B. bufo* samples were obtained from Birkdale sand dunes (Merseyside); *B. calamita* were also obtained from Haverigg dunes (Cumbria) and *B. bufo* from a field pond near Brighton (Sussex) . In some cases animals of known parentage (and thus species) were used, in others a preliminary classification was made based on morphological characters. From each larva a 2 mm section of tail tip (< 2 mg) was removed by scalpel and stored immediately in an eppendorf tube containing 0.5-1.0 ml pure ethanol. These samples were kept for at least six months, at environmental temperatures, prior to analysis.

Standard reagents for protein electrophoresis and silver-staining, including molecular weight markers, were purchased from Sigma Chemicals, Poole, UK. Molecular biology grade agarose and DNA molecular weight markers (1 kb ladder) were from Gibco-BRL (UK), *Taq* DNA polymerase was from Genpak (UK), and 10-mer oligonucleotide primers were generated by the University of Sussex DNA synthesizer. Chelex 100 resin was from Bio-Rad, Richmond, California.

PROTEIN TYPING

Each tail tip was heated at 65°C for 10 min in 60 µl loading buffer (50 mM Tris-HCl pH 8, 0.15 M b-mercaptoethanol, 1% sodium dodecyl sulphate [SDS], 10% glycerol, 0.01% phenol red), homogenized by gentle pipetting, and immersed in a boiling water bath for 2 min. Solid debris was removed by centrifugation at 1000 x g for 25 seconds, and 40 µl supernatant then loaded into each gel well.

A stacking gel of 4% acrylamide, 0.2% bisacrylamide and 10% glycerol in 125 mM Tris-HCl pH 6.8, 0.1% SDS was used with a separating gel of 7.5% acrylamide, 0.2% biscarylamide in 0.38 M Tris-HCl pH 8.8, 0.1% SDS. Electrophoresis was for about 5 hr, at 50 v through the stacking gel and 100 v through the separating gel. Proteins were then fixed by immersing the gel in 50% (v/v) methanol, 10% (v/v) glacial acetic acid for 2 hours, then overnight in 50% methanol alone.

Proteins were silver-stained (Switzer *et al.*, 1979) by immersing the gel for 15-20 min in saturated ammoniacal silver nitrate solution (0.8% silver nitrate made up in 0.08% NaOH and 0.035% ammonium hydroxide). Excess silver nitrate was removed by washing in dis-

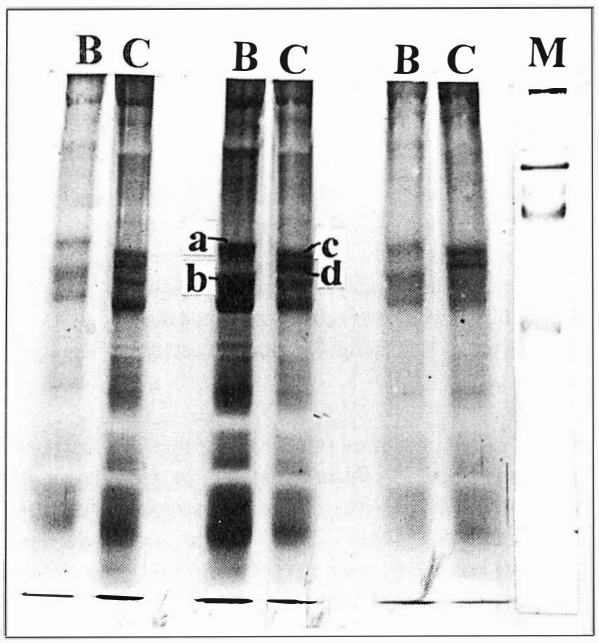

FIG. 1. Identification of *Bufo* larvae by protein fingerprinting. Larval tail tip proteins from three individuals of each species were electrophoresed and stained as described in Methods. B: *Bufo bufo*; C: *Bufo calamita;* M: Molecular weight markers; a, b: distinctive *B. bufo* bands; c, d: distinctive *B. calamita* bands. Molecular weight markers visible on this gel (a composite photo with the marker lane cut and moved next to the samples) were egg albumin (45,000), bovine plasma albumen (66,000), phosphorylase B subunit (97,400) and b-galactosidase subunit (116,000).

tilled water for 5-10 min, and stain developed by the addition of 0.0046% citric acid, 0.00185% formaldehyde. The reaction was stopped by immersing the gel in 50% methanol.

RAPD ANALYSIS

DNA was extracted from tail tips by incubating each at 55°C overnight in 160 µl sterile distilled water (SDW) with 40 µl of a Chelex 100 resin suspension made up in SDW. Each sample was then briefly vortex-mixed, immersed in a boiling water bath for 8 min, vortexed again and centrifuged at 8000 x g for 3 min. 1 µl aliquots of the supernatants were then used in polymerase chain reactions (PCRs).

Each PCR was in a final volume of 20 µl and included 1 µl DNA extract, 50 mM Tris-HCl pH 8.5, 16 mM ammonium sulphate, 0.15 mg/ml bovine serum albumin, 3.5 mM $MgCl_2$, 0.1 mM dATP, dGTP, dCTP and dTTP, 0.2 mM oligonucleotide and 0.4 units Genpak *Taq* polymerase. An initial denaturation cycle (94°C x 4 min) was followed by 35 cycles each of: 94°C x 1 min, 40°C x 1 min, 72°C x 2 min, followed by a final extension cycle of 72°C x 4 min.

Each sample was mixed with 5 µl loading buffer (60% w/v sucrose, 2.5 mg/ml bromophenol blue, in 2 x RB; RB [running buffer] = 13.5 mM Tris-acetate pH 8.3, 0.3 mM EDTA) and electrophoresed through 1.5 % agarose in RB containing 1 µg/ml ethidium bromide. After electrophoresis at 60 v for 2-3 hours the gel was examined by UV transillumination and photographed.

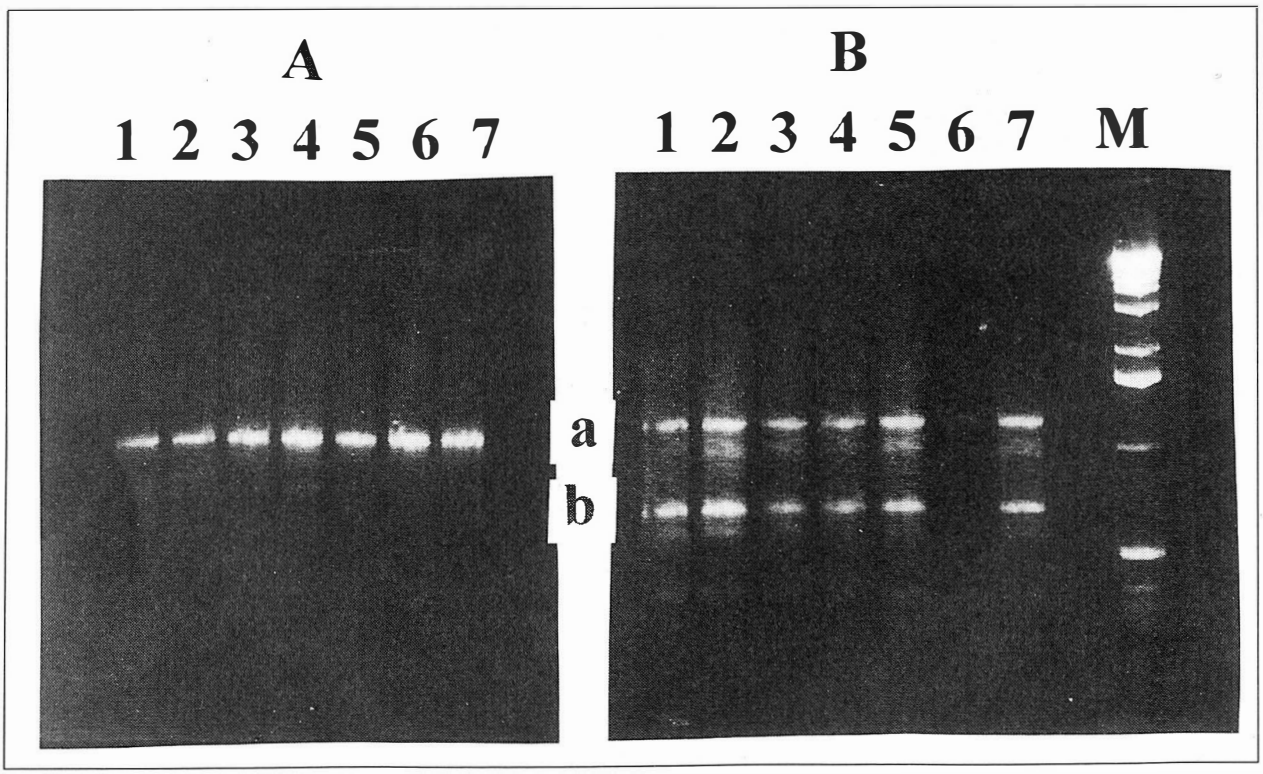

FIG. 2. Identification of *Bufo* larvae by RAPD analysis. Larval tail tip DNA from seven individuals of each species was amplified by PCR using primer PR4 and products identified by ethidium bromide staining after electrophoresis, all as described in methods. A: *B. calamita*; B: *B. bufo*; a: common PCR product; b: *B. bufo*-specific PCR product; M: 1 kb ladder molecular size markers.

RESULTS

PROTEIN TYPING

A representative sample of three *B. bufo* and three *B. calamita* tail tip protein profiles is shown in Fig. 1. The kinetics of silver-staining were highly sensitive to protein concentration, and the inevitable small variations between samples generated different optimal "stop times" for the reactions in the various gel lanes. Good quality photography of such gels is therefore difficult. Nevertheless, because the gels were observed carefully during the staining reaction, it was possible to score each individual at its optimum staining point. *B. bufo* larval tail tips exhibited a distinctive band pair (Fig. 1a,b) of approximate molecular weights 58 900 and 50 100, while *B calamita* larvae had an intermediate pair (Fig. 1c,d) of about 57 500 and 53 700 daltons. The relative gap sizes between these bands were very distinctive and quickly recognizable during stain development.

Reliability of the procedure was ascertained in two ways. Firstly, screening many tens of individuals of known parentage from geographically distant populations never revealed a single anomalous individual. Secondly, a blind test was carried out: three individual tail tips of each species, of known parentage, were ascribed random numbers by one of us and analysed by another. All six were correctly identified by the experimenter.

A sample of 400 larval tail tips from ponds where competition between the two species was under study was then subjected to protein analysis, after individual tadpoles were given a preliminary identification on morphological grounds. The results are shown in Table 1. Only 1% did not yield gel banding patterns clear enough to score; however, the data suggest that 11.4% of putative *B. bufo* and 13.4% of putative *B. calamita* were misclassified on morphological criteria (mainly relative size and chin patch occurrence).

RAPD ANALYSIS

Eight 10-mer primers were screened initially with DNA samples from *B. bufo, B. calamita* and *Rana temporaria* larvae. One of these primers (PN4, 5'-GCAAGTAGCT-3') yielded simple and apparently species-specific electrophoretic phenotypes. This primer was tested with DNA from seven individuals of each species, again taken from different populations. The results are shown in Fig. 2, in which only one individual (*B. bufo* no. 6) failed to yield amplification products. PN4 yielded two main products, one of about 1.24 kb common to both species (band a), and one of about 0.79 kb found only in *B. bufo* samples.

DISCUSSION

In this study we have developed two molecular methods for the identification of larvae of two species of *Bufo*. Both seem to be equally sensitive and take similar amounts of time. Tail tips can be taken from the smallest tadpoles (Gosner stage 26: Gosner, 1960) with very low risk of mortality. Except for the smallest (immediately post-hatch) *B. calamita* larvae, which experienced death rates higher than controls after tail-tip amputation, survivorship was unaffected by the excision when larvae were subsequently reared in the laboratory (data not shown). In the field, larvae were normally retained for a few hours prior to release to allow wound healing and thus minimize the danger from predators responding to olfactory cues arising from tissue damage. Protein typing benefited from greatly increased sensitivity using silver stain compared with coomassie blue (Beebee, 1990), and from the demonstration that tissue for protein analyses of this kind can be preserved conveniently in ethanol rather than at low temperatures. Protein typing requirements are therefore less stringent than those for allozyme studies, in which enzyme activity must be retained and the proteins preserved in a non-denatured state.

We expect these techniques to be useful for fieldworkers studying *B. bufo* and *B. calamita*, and that they will be readily extrapolated to other species combinations. We have, for example, subsequently shown that RAPD primer PN4 also distinguishes larvae of *B. viridis* from those of *B. bufo* and *B. calamita,* and is thus a specific indicator for all three European *Bufo* (data not shown). Tail tips can be collected quickly from large numbers of larvae, permitting retrospective identification after subsequent laboratory analysis. It is of course important always to run control samples of known species on every gel, but each gel can (depending on mould size) also take 10-20 unknowns simultaneously. Protein typing is cheaper than PCR-based methods and requires less specialised equipment; conversely, ethidium bromide staining is simpler than silver staining and DNA extracts can be used for other purposes, such as amplification and sequencing of specific genes of interest. The choice between them will therefore usually be dictated by available facilities and the details of particular research objectives.

TABLE 1. Comparative identification of *Bufo* larvae by morphology and protein fingerprinting.

Species predicted from morphology	No. identified as *B. bufo* by fingerprinting	No. identified as *B. calamita* by fingerprinting	No. indistinguishable by fingerprinting
Bufo bufo	225	19	2
Bufo calamita	29	123	2

ACKNOWLEDGMENTS

We thank David Simpson and Paul Rooney for help at fieldwork sites, Dr Susan Hitchings for provision of some *B. bufo* larvae, Dr Sandy MacGillivray for advice about silver staining, and the Leverhulme Trust for financial support.

REFERENCES

Banks, B. & Beebee, T. J. C. (1987). Spawn predation and larval growth inhibition as mechanisms for niche separation in anurans. *Oecologia* **72,** 569-573.

Beebee, T. J. C. (1977). Environmental change as a cause of natterjack toad (*Bufo calamita*) declines in Britain. *Biol. Conserv.* **11,** 87-102.

Beebee, T. J. C. (1990). Identification of closely-related anuran early life-stages by electrophoretic fingerprinting. *Herpetol. J.* **1,** 454-457.

Davis, C. A. (1985). *The population dynamics of the natterjack toad (Bufo calamita, Laurenti) in the North Merseyside sand-dune system.* PhD thesis, Liverpool Polytechnic.

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16,** 183-190.

Heusser, H. (1972). Intra- und interspezifische Crowding-effekte bei Kaulquappen der Kreuzkrote, *Bufo calamita* Laur. *Oecologia* **10,** 93-98.

Kimberling, D. N., Ferreira, A. R., Shuster, S. M. & Keiss, P. (1996). RAPD marker estimation of genetic structure among isolated northern leopard frog populations in the south-western USA. *Mol. Ecol.* **5,** 521-529.

Masters, B. S. (1995). The use of RAPD markers for species identification in Desmognathine salamanders. *Herpetol. Rev.* **26,** 92-95

Masters, B. S. & Forester, D. C. (1995). Kin recognition in a brooding salamander. *Proc. Royal Soc. B.* **261,** 43-48.

Smith, M. A. (1951). *The British Amphibians and Reptiles.* Collins, London.

Switzer, R. C., Merril, C. R. & Shifrin, S. (1979). A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal. Biochem.* **98,** 231-237.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nuc. Acids Res.* **18,** 6531-6535.

Accepted: 14.11.97