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# IDENTIFICATION OF CLOSELY-RELATED ANURAN EARLY LIFE-STAGES BY ELECTROPHORETIC FINGERPRINTING

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## ABSTRACT

(1) Protein electrophoretic patterns from early life stages of two anurans, *Bufo bufo* and *Bufo calamita*, were compared. Samples were taken from two widely-separated populations of each species.

(2) Spawn jelly of the two toads could be distinguished reliably by the difference in molecular weight of a single major component (30,000 in *B. bufo*, 35,000 in *B. calamita*).

(3) Small tadpole total proteins could be distinguished by the presence (*B. bufo*) or virtual absence (*B. calamita*) of two characteristic proteins of Mrs 49,000 and 53,000.

(4) Large tadpole tailfin proteins also differed in a species-specific way; *B. bufo* exhibited a strong band of Mr 62,000 that was almost absent in *B. calamita*, while the latter species had a doublet (Mrs 57,000 and 58,000) instead of the single 57,000 Mr polypeptide seen in *B. bufo*.

## INTRODUCTION

Molecular analyses offer great improvements in many areas of taxonomic biology where, in the past, there has been almost total reliance on morphological criteria. The more precise molecular approach has many possible uses, including service to field studies in cases where there are difficulties in recognising closely-related species. A good example of this situation arises within the amphibia; in Britain the two species of toad *Bufo bufo* and *Bufo calamita* are readily distinguished as adults, but with much greater difficulty as eggs and larvae. Morphological criteria for spawn and tadpoles do exist (e.g. see Smith, 1951; Beebee, 1983) but are notoriously unreliable. Larvae of both species, for example, are uniformly black and of very similar shape; identification is currently based on difficult measurements of mouth-width or tooth patterns, or on the appearance later in development of a white 'chin patch' in *B. calamita*. The first two of these criteria are very time-consuming on large samples, and all three have substantial error rates (e.g. Mathias, 1971; Beebee, 1977).

In this paper I describe a novel molecular approach to the identification of toad eggs and larvae that, for the above two species, has proved completely reliable.

## MATERIALS AND METHODS

### MATERIALS

Small samples of spawn and tadpoles of both toads were collected (that of *B. calamita* under licence) from two widely separated sites (Hampshire and Cumbria) in England; development was sustained thereafter in the laboratory (Beebee and Beebee, 1978). Routine chemicals, including electrophoresis-grade acrylamide, were from BDH (Poole, UK) and molecular weight marker proteins (range: 29,000-205,000) were from Sigma (Poole, UK).

### SAMPLE PREPARATION

0.5-1.0ml batches of spawn jelly were cut and detached from spawn strings using scissors and forceps, and stored until needed at -20°. Each sample was then lyophilised overnight, dissolved in 100-200$\mu$l loading buffer (LB = 50mM Tris-HCl pH 6.8, 0.15M merceptoethanol, 1% SDS, 10% glycerol, 0.001% phenol red) and immersed in a boiling water bath for 5 minutes. 50$\mu$l were used immediately after cooling for electrophoretic analysis. Small (<12mm) tadpoles were killed by flash-freezing, suspended in 100$\mu$l LB and treated as above prior to electrophoresis of 10-50$\mu$l aliquots. Sections of dorsal tailfin were rapidly exised from large (12-25mm) larvae laid on a damp tile, using a razor to cut as shown in Fig. 1. Tadpoles were returned to the tanks, and the fin sample suspended in 100$\mu$l LB followed by the same treatment employed for entire small tadpoles.

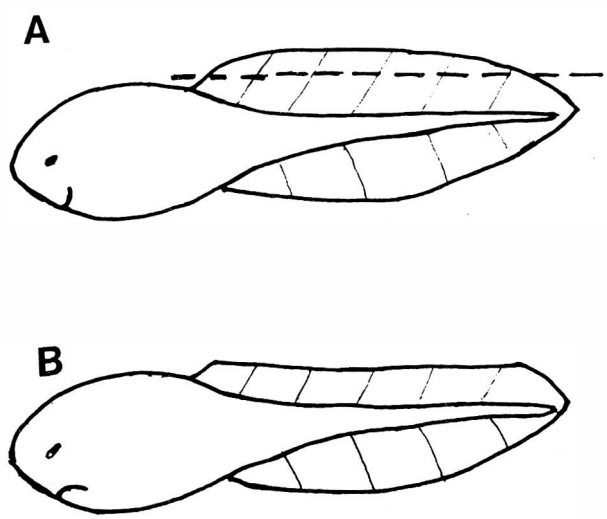


Fig. 1 Tailfin clipping regime.

ELECTROPHORESIS

This was carried out under strictly denaturing conditions. Separating slab gels contained 7.5-12% acrylamide, 0.2-0.35% bis acrylamide made up in 0.33M Tris-HCl pH 8.8, 0.1% SDS. A stacking gel of 5% acrylamide, 60mM Tris-HCl pH 6.8, 0.1% SDS was used and a running buffer of 50mM Tris, 0.38M glycine, 0.1% SDS pH 8.3. Electrophoresis was normally for about 6 hours at 100V. Staining was for 1-2 hours in 0.1% Coomassie blue in 10% acetic acid, 50% ethanol, followed by overnight destaining in 10% acetic acid. Gels were then photographed and, if required, scanned densitometrically using a LKB ultroscan with laser beam.

## RESULTS

Examples of spawn jelly protein separations are shown in Fig. 2. All *B. calamita* samples showed strong bands at or about Mr 35,500 (band A on figure) whereas *B. bufo* spawn always showed a band of higher mobility (Mr around 30,000 position B). These were the strongest bands visible in the jelly extracts, and could be seen easily even in old spawn at the point of hatch (i.e. up to 14 days after deposition) although there was evidence of substantial degradation at these later times. The distinction was highly reliable, with similar patterns evident in spawn samples from

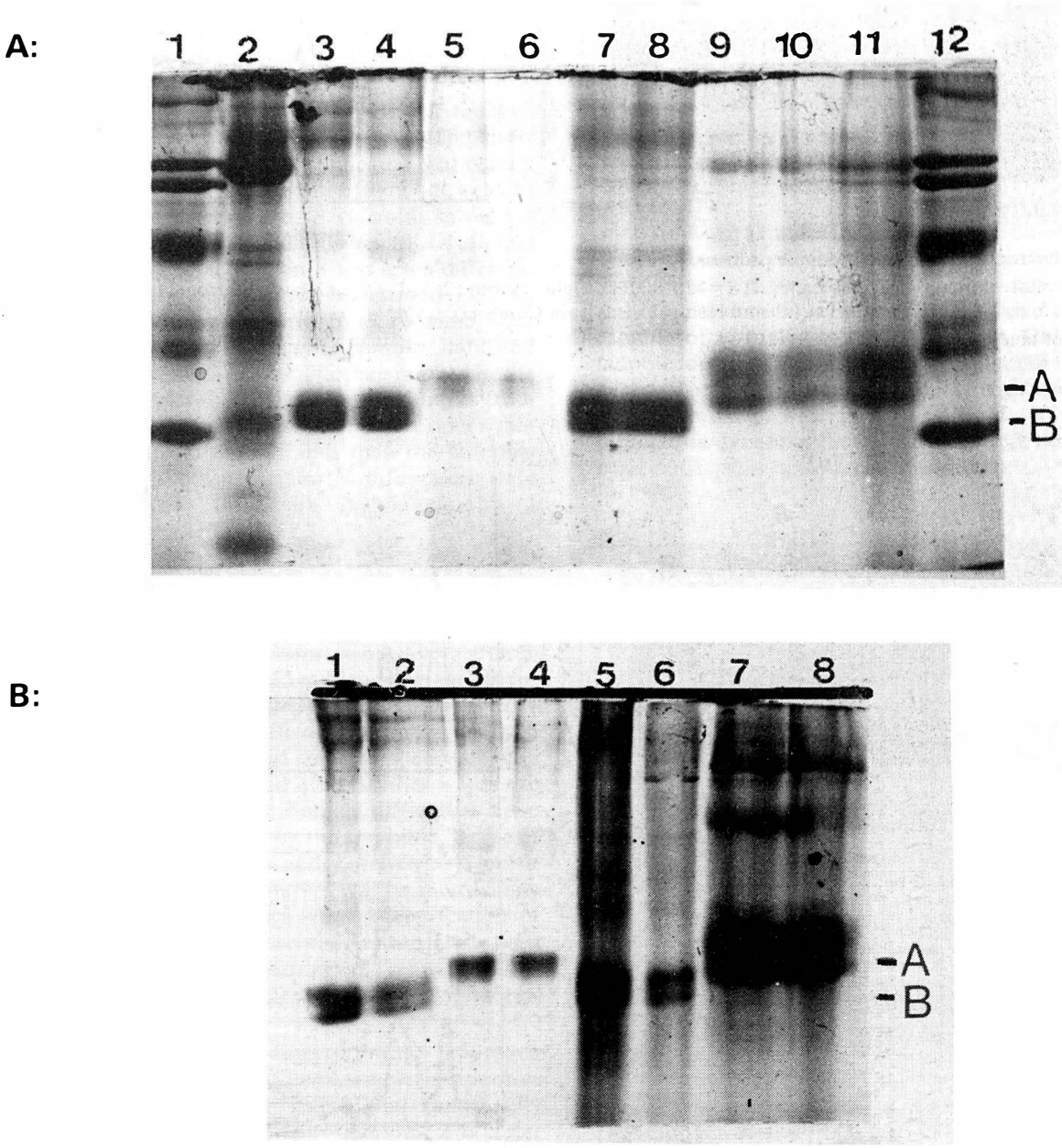


Fig. 2 Electrophoresis of spawn jelly proteins.

A: Samples from Hampshire populations on a 10% acrylamide gel. Lanes: 1, 12 = molecular weight markers; 2-*Rana temporaria*; 3-4 = fresh laid *B. bufo* spawn; 5, 6 = fresh laid *B. calamita* spawn; 7, 8 = *B. bufo* spawn at point of hatch; 9, 10, 11 = *B. calamita* spawn at point of hatch.

B: Samples from Cumbria populations on 12% gel. Lanes: 1, 2 = fresh *B. bufo* spawn; 3, 4 = fresh *B. calamita* spawn; 5, 6 = hatching *B. bufo* spawn; 7, 8 = hatching *B. calamita* spawn.

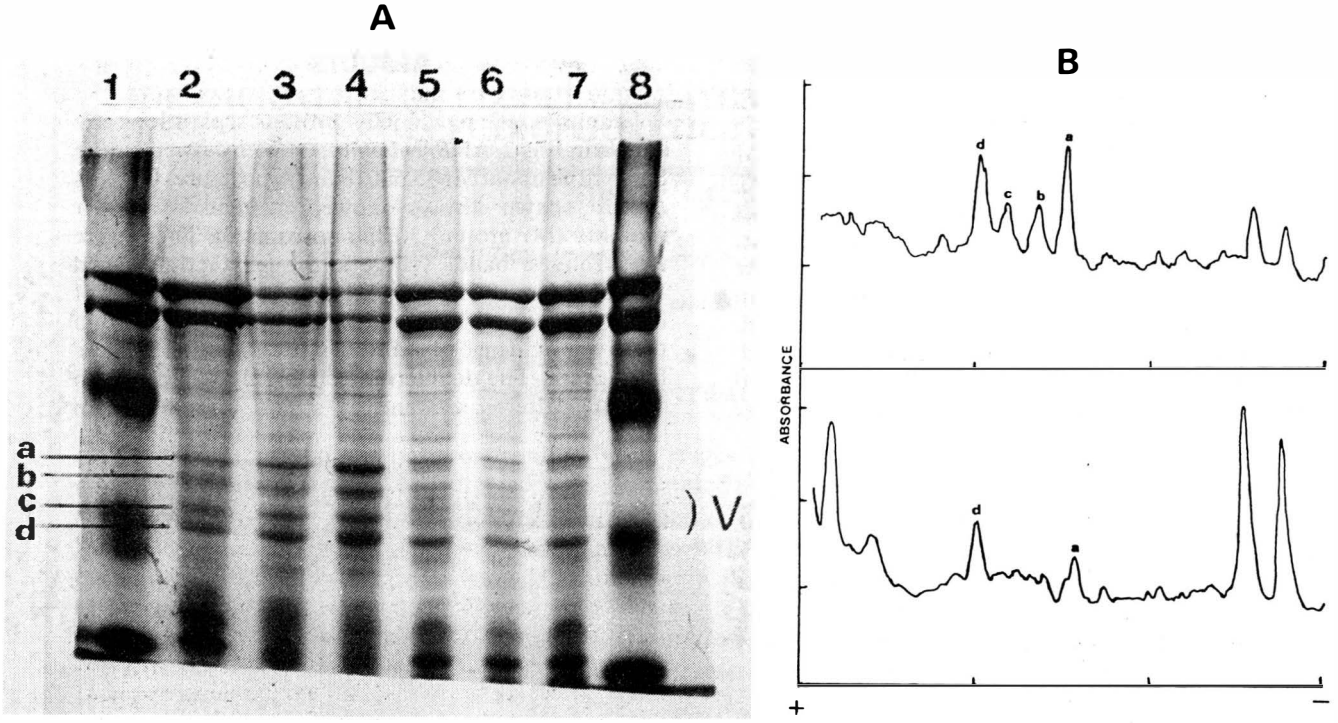


Fig. 3 Electrophoresis of small tadpole proteins.

A: Electrophoretogram (7.5% gel). Lanes: 1, 8 = Molecular weight markers; 2, 3, 4 = *B. bufo* larvae (2 from Hampshire, 1 from Cumbria); 5, 6, 7 = *B. calamita* larvae (1 from Hampshire, 2 from Cumbria).

B: Scans of lanes 4 (above) and 5 (below).

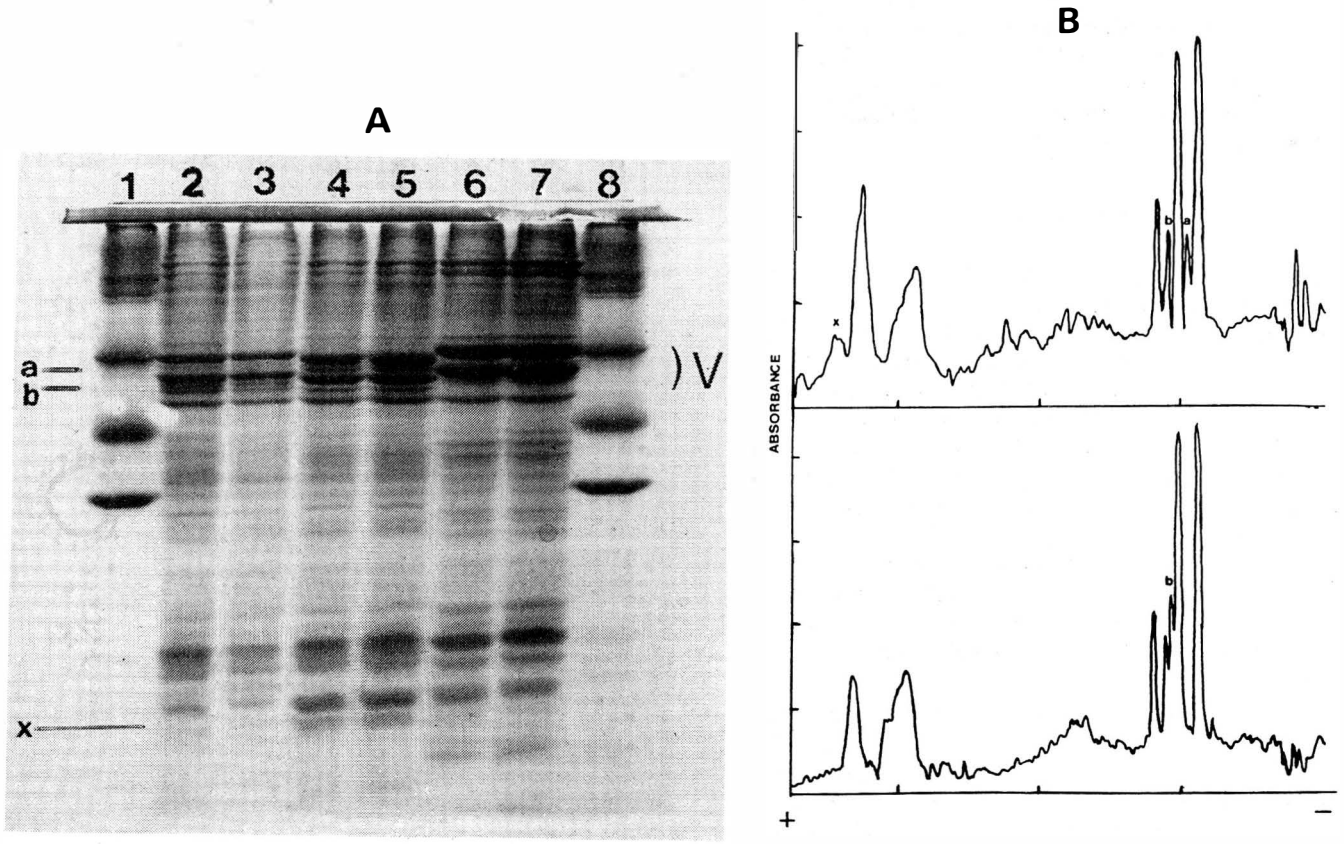


Fig. 4 Electrophoresis of large tadpole tailfin proteins.

A: Electrophoretogram (12% gel). Lanes: 1, 8 = Molecular weight markers (different from those used in Figs. 2 and 3); 2, 3 = *B. calamita* tailfins; 4, 5 = *B. bufo* tailfins; 6, 7 = *R. temporaria* tailfins. In each case one specimen was from Hampshire and the other from Cumbria.

B: Scans of lanes 4 (above) and 3 (below).

northern and southern England. Spawn jelly from a more distantly related anuran, *Rana temporaria* (lane 2) was quite different and showed a more complex banding pattern than the bufonid material.

Total protein patterns from three *B. bufo* and three *B. calamita* small tadpoles are shown in Fig. 3A. Again the patterns were generally reproducible, though the relative intensities of the high molecular weight proteins (90,000-120,000) and some very small ones varied substantially between tadpole extracts. Of greatest value was the region V, in which 4 bands a, b, c and d were always clearly discernible in *B. bufo* whereas b and c were indistinct or absent in all *B. calamita* samples. The presence or absence of strong b and c bands (Mrs 53,000 and 49,000), relative to a and d, was apparently diagnostic; differences in this part of the gel were confirmed by densitometer tracing (Fig. 3B).

Tailfin proteins from larger tadpoles of the two species were different again (Fig. 4A). The most reliable area of comparison, V, indicated that band a (Mr 62,000) was always strong in *B. bufo* but weak in *B. calamita*; and in region b, *B. calamita* showed a doublet (Mrs 57,000 and 58,000) whereas *B. bufo* had just a singlet of 57,000. Region X was often different, with a dense band of estimated Mr around 7,000 in *B. bufo* and little or nothing in *B. calamita*, but comparison of larger numbers of samples indicated that this difference was less reliable than implied by the particular examples shown here. Tailfin samples from *Rana temporaria* larvae (lanes 6 and 7) were once again more different from the bufonids than the bufonids were from each other. Region V differences were totally conserved; these are highlighted by a densitometer trace (Fig. 4B).

## DISCUSSION

*Bufo bufo* and *Bufo calamita* probably diverged from a common ancestor at least 15 million years ago (Blair, 1971; Beebee, 1983). Nevertheless, their early developmental stages remain difficult to distinguish on morphological grounds and this poses considerable problems for ecological and other studies where the two species are sympatric. Similar difficulties arise with other species (e.g. among the amphibia, larvae of the newts *Triturus vulgaris* and *T. helveticus*), and it seems that molecular techniques will be increasingly useful to population studies where identification of large numbers of larvae is essential. It has already been shown that eggs of European newts (genus *Triturus*) can be identified by soluble protein electrophoretograms (Veith, 1987).

The electrophoretic fingerprinting approach described here is reasonably quick, simple, inexpensive and precise. It is easy to collect 1ml spawn jelly samples, or individual small tadpoles, for later analysis; even tailfin sections can be taken in less than a minute. The whole electrophoretic analysis, from the start of lyophilisation (only necessary with spawn, where protein is too dilute to run directly) to destaining, takes less than 36 hours and large numbers of samples (at least 12 per gel) can be processed simultaneously. Identification can be by inspection; photography or scanning is only required if a permanent record is needed. The chemicals are all cheap and the necessary apparatus present in most reasonably-equipped laboratories. In the multiple samples studied so far, all of known parentage, not a single ambiguity has arisen with respect to identification after examination of tens of specimens and the differences seem well conserved between populations hundreds of kilometres apart. Some bands, however, were more erratic in intensity between individuals than others and these were excluded from use in identification. The reasons for this variability were not clear but I suspect reflect different protein solubilisation efficiencies rather than real differences between individuals; the variation was usually greatest with the high Mr proteins, which may be less soluble or more prone to aggregation (thus not entering the gel properly). As a general rule it would seem wise to include reference samples of known parentage in such analyses when samples from the field are under study. Finally, much of the work can be done non-destructively; taking spawn jelly does not damage embryos, and tailfin clipping leaves viable tadpoles capable of virtually normal swimming. Only small tadpole identification involves mortality, but this is at a stage where abundance is usually so high that small-scale sampling will have negligible effects on population dynamics (both toads lay more than 1,000 eggs, natterjacks up to more than 7,000).

## ACKNOWLEDGEMENTS

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## REFERENCES

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

Beebee, T. J. C. (1983). The Natterjack Toad. Oxford University Press.

Beebee, T. J. C. and Beebee, M. L. (1978). A quantitative study of metamorphosis in the natterjack toad, *Bufo calamita*. *British Journal of Herpetology*. **5**, 689-693.

Blair, W. F. (1971). Ed. Evolution in the genus *Bufo*. University of Texas, Austin.

Mathias, J. H. (1971). Ecology of two species of amphibian (*Bufo bufo* and *Bufo calamita*) on the Ainsdale Sand Dunes National Nature Reserve. *PhD thesis*, University of Manchester.

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

Veith, M. (1987). Egg and embryo proteins in European newts (genus *Triturus*) and their taxonomic potential. *Amphibia-Reptilia.* **8**, 203-211.