Application of Isoelectric Focusing in Molluscan Systematics

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ABSTRACT

The application of isoelectric focusing (IEF) in molluscan systematics is reviewed and illustrated using literature data and unpublished analyses. IEF can be used as any other electrophoretic method, but is most appropriate for: (1) generating complex species-specific banding profiles, (2) assessing overall genetic similarities, (3) supplementing conventional electrophoretic techniques by resolving hidden protein variation and (4) investigating minute organisms.

Key Words: Mollusca; systematics; phylogeny; population genetics; protein electrophoresis; isoelectric focusing.

INTRODUCTION

Protein electrophoresis is still one of the most frequently used molecular techniques in systematics and population genetics. The basis for this technique is that mobility differences of proteins in an electric field reflect changes in their amino acid composition and thus mirror differences at the gene level. Hence, it is a simple, indirect, way to look at gene pools.

However, as conventional electrophoretic methods only detect mobility or molecular weight differences, they may fail to resolve hidden protein heterogeneity caused by amino acid replacements that are not accompanied by substantial charge and/or molecular mass alterations (Coyne *et al.*, 1979; Ramshaw *et al.*, 1979; Singh, 1979; Ferguson, 1980).

Other separation methods such as Isoelectric Focusing (IEF), may reduce this problem. IEF separates proteins according to their isoelectric point (pI) (*e.g.*, Righetti, 1983). To this end one creates a pH gradient by electrophoretic segregation of "carrier ampholytes" (*i.e.* synthetic polyaminopolycarboxylic acids) in a supporting medium. Proteins placed in such a pH gradient will move according to their net charge until they reach a point where the pH equals their pI so that their net charge becomes zero and no further migration occurs. In this way, IEF can separate protein fractions with pI values differing by only 0.01 pH units (Drysdale, 1975; Righetti, 1983). Such resolution by charge is not normally obtainable by other electrophoretic methods and IEF is therefore well suited to examine hidden heterogeneity (Drysdale, 1975; Ross, 1977; Righetti, 1983; Cicchetti *et al.*, 1990).

In this paper we review the use of IEF in molluscan systematics. We therefore provide a survey of IEF applications, after which we focus on IEF data treatment insofar as this differs from other electrophoretic techniques. For general technical accounts on the method we refer to Righetti (1983) and Whitmore (1990a), even though we present some basic guidelines in Appendix 1. Authorships of the molluscan taxa mentioned are provided in Appendix 2.

REVIEW OF IEF APPLICATIONS IN MOLLUSCAN SYSTEMATICS

Gastropoda: Pulmonata

The first applications of IEF in molluscan systematics we could trace, were published by Saladin *et al.* (1976) who used general egg proteins to distinguish between the bulinids *Bulinus liratus* and *B. obtusispira* from Madagascar. Ross (1977) studied glucose phosphate isomerase patterns in *Bulinus* spp. but drew no conclusions. Subsequently, Rollinson and Southgate (1979) investigated five enzymes in 38 populations of four *B. africanus* group

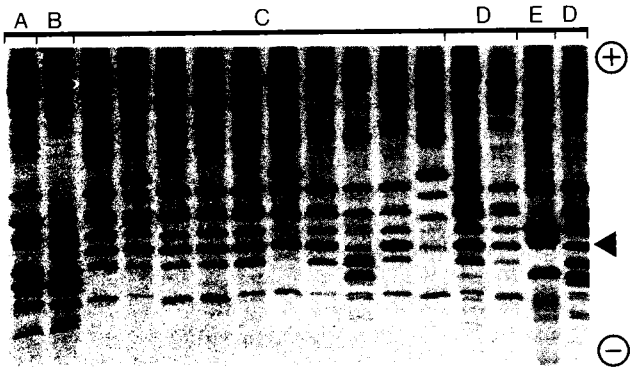


Figure 1. Agarose IEF (pH 4–6.5) of digestive gland esterases in the *Arion hortensis* complex. A-D: *A. distinctus* (A: Brasschaat; B: Wilrijk; C: Hoogstraten; D: Wilrijk). E: *A. hortensis* (Wilrijk).

species in Tanzania and found that *B. nasutus* is clearly differentiated from the other three species. In a more extensive survey of eight of the ten nominal species in the *B. africanus* group, Wright and Rollinson (1979) noted that certain enzyme profile combinations appeared to be associated with some taxa and others with regional distributions. Wright *et al.* (1979) found little heterogeneity within and between populations of *B. senegalensis* (based on five enzymes), but snails parasitized by different trematodes were easily distinguished. Wright and Rollinson (1981) investigated the same five enzymes in 103 populations of the *B. tropicus-truncatus* complex and found that diploid and tetraploid populations were clearly different. These observations were used by Brown and Rollinson (1982) and Brown *et al.* (1982) to characterize *B. truncatus* in the southern part of its distribution and to show that *B. coulboisi* from Lake Tanganyika is only a southern form of *B. truncatus*. Similarly, Brown *et al.* (1986) used IEF enzyme profiles to show that *B. guernei* from West Africa is conpecific with *B. truncatus*, while Southgate *et al.* (1985, 1989) relied on Wright and Rollinson's (1979, 1981) work to demonstrate that diploid Kenyan populations of *B. tropicus* can transmit the fluke *Schistosoma bovis* and that snails parasitized by different trematodes can be separated on the basis of their IEF profiles. Rollinson and Wright (1984) and Rollinson *et al.* (1990) surveyed several enzyme loci in *B. cernicus* from Mauritius. Allele frequencies at these loci showed clear spatial heterogeneities, but were remarkably consistent over a period of six years. Finally, Brown and Shaw (1989) and Brown *et al.* (1991) used IEF of five enzymes to separate Kenyan *B. tropicus*, *B. truncatus* and *B. permembranaceus*.

Backeljau (1985) conducted an IEF analysis of esterases in sibling species of the *Arion hortensis* complex (Figure 1). Mean intra- and interspecific band similarity values showed that *A. hortensis*, *A. distinctus* and *A. owenii* are clearly different. The same study also illustrated the striking difference between monomorphic IEF profiles of uniparental species (*e.g.*, *A. intermedius*) and the highly variable profiles of allogamous species (*e.g.*, *A. hortensis* and *A. distinctus*). Because of this, Backeljau (1985) assumed that *A. owenii* might be a facultative uniparental species. However, the specimens investigated were probably highly inbred for they belonged to a captive stock derived from the original material used by Davies (1977, 1979). Hence the lack of variation in these profiles may have been caused by sustained inbreeding as well. Finally, since the IEF profiles of *A. owenii* were very similar to those of *A. intermedius*, Backeljau (1985) suggested that the latter species belongs to the same subgenus as *A. hortensis* s.l. This conclusion was further elaborated by Backeljau and De Bruyn (1990). In a similar way Backeljau *et al.* (1987) dealt with the *A. fasciatus* complex (Figure 2). IEF profiles of albumen gland proteins and esterases clearly separated three presumed species: *A. fasciatus*, *A. circumscriptus* and *A. silvaticus*. In contrast to *A. hortensis* and *A. distinctus*, but comparable to *A. intermedius*, *A. fasciatus* s.l. revealed a remarkable "intraspecific" profile constancy (Figure 2), even over large geographic distances. This was inter-

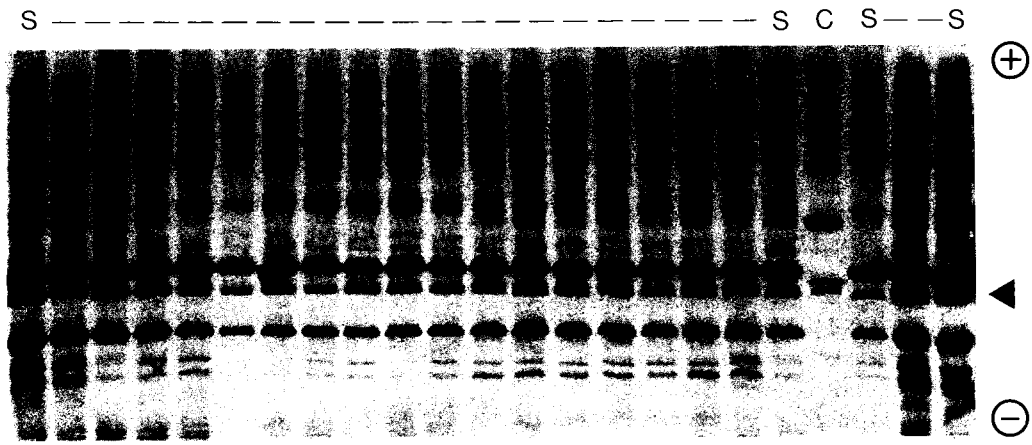


Figure 2. Agarose IEF (pH 4–6.5) of digestive gland esterases in *Arion circumscriptus* (C) and *A. silvaticus* (S).

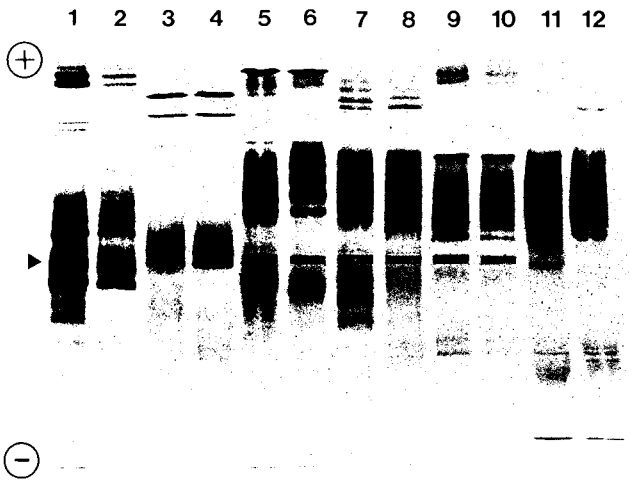


Figure 3. Detection of a cryptic species (genus *Arion*, subgenus *Kobeltia*) by agarose IEF of albumen gland proteins in a 4–6.5 pH gradient. 1–2: *A. (K.) fagophilus* (Alsasua, Spain); 3–4: *A. (K.) intermedius* (3. Boeckhoute, Belgium; 4. Hamburg, Germany); 5–6: *A. (K.) distinctus* (Deurne, Belgium); 7–8: unidentified *A. (K.) hortensis* like species from southern France; 9–10: *A. (K.) hortensis* (Wilmslow, U.K.); 11–12: *A. (K.) owenii* (11. Buncrana, Ireland; 12. London, U.K.).

preted as indicating uniparental reproduction and therefore Backeljau *et al.* (1987) suggested to consider *A. fasciatus* s.l. as an agamospecies complex. A weight analysis suggested that the putative albumen gland protein polymorphism in *A. circumscriptus* was not due to developmental differences. The limited esterase variation, on the contrary, was assumed to be environmentally or physiologically determined (*e.g.*, Oxford, 1975, 1978). Backeljau and De Winter (1987), finally, characterized albumen gland protein profiles of two paratypes of *A. fagophilus* in a qualitative IEF comparison of 10 arionid species. This work revealed a fundamental difference in the albumen gland proteins of the subgenera *Kobeltia* and *Carinarion* on the one hand, and *Arion* and *Mesarion* on the other. A review of the use of albumen gland proteins in arionid systematics was presented by Backeljau (1989). In this context, Figure 3 shows an unpublished comparison of albumen gland profiles of six arionids, indicating that an *Arion (Kobeltia) hortensis*-like slug from southern France differs so much from three morphologically extremely similar species (*A. (K.) hortensis*, *A. (K.) distinctus* and *A. (K.) owenii*), that it probably belongs to another (undescribed?) species.

In order to supplement morphological observations Manga-Gonzalez and Rollinson (1986) surveyed five enzymes to differentiate seven *Helicella* species. Two enzymes, malate dehydrogenase and glucosephosphate isomerase, were sufficient to separate all taxa.

Brito (1992) conducted a preliminary qualitative IEF analysis of esterases in seven species of Zonitidae, representing three genera and three subgenera. This work showed that IEF is also useful for taxonomic purposes in this group.

Gastropoda: Prosobranchia

Using IEF of esterases and general proteins Sella and Badino (1980) demonstrated that Mediterranean *Patella coerulea* and *P. aspera* are distinct, yet closely related, species, while *P. lusitanica* is very different (nomenclature used by Sella & Badino, 1980).

In order to find taxon specific IEF profiles, Viyanant *et al.* (1985) analyzed 12 specific enzymes in two species and one subspecies of *Bithynia* in Thailand. Their work showed that *B. funiculata* and *B. siamensis siamensis* differ consistently in four enzymes, while the subspecies *B. siamensis siamensis* and *B. siamensis goniomphalos* only differ in their esterase profiles.

Unpublished preliminary IEF patterns of esterases and general proteins of *Baicalia* species from Lake Baikal (Russia) illustrate the performance of automated IEF using PhastSystem (see Appendix 1). Figure 4 shows interpopulation esterase heterogeneity in *B. costata*, while Figure 5 compares esterase profiles of *B. costata* and *B. turriformis*. Genetic variation at a monomeric, diallelic esterase locus in this latter species is illustrated in Figure 6, while Figure 7 shows the monomorphic profiles of *B. bithyniopsis*. Finally, two presumed species in the *B. herderiana* complex, *viz. B. ventrosula* and *B. herderiana laevis*, reveal variable general protein profiles, but little or no interspecific differentiation (Figure 8).

Qualitative IEF profiles were also used by Nyumura and Hosokawa (1993) to separate two morphotypes of the apple snail *Pomacea canaliculata* in Japan.

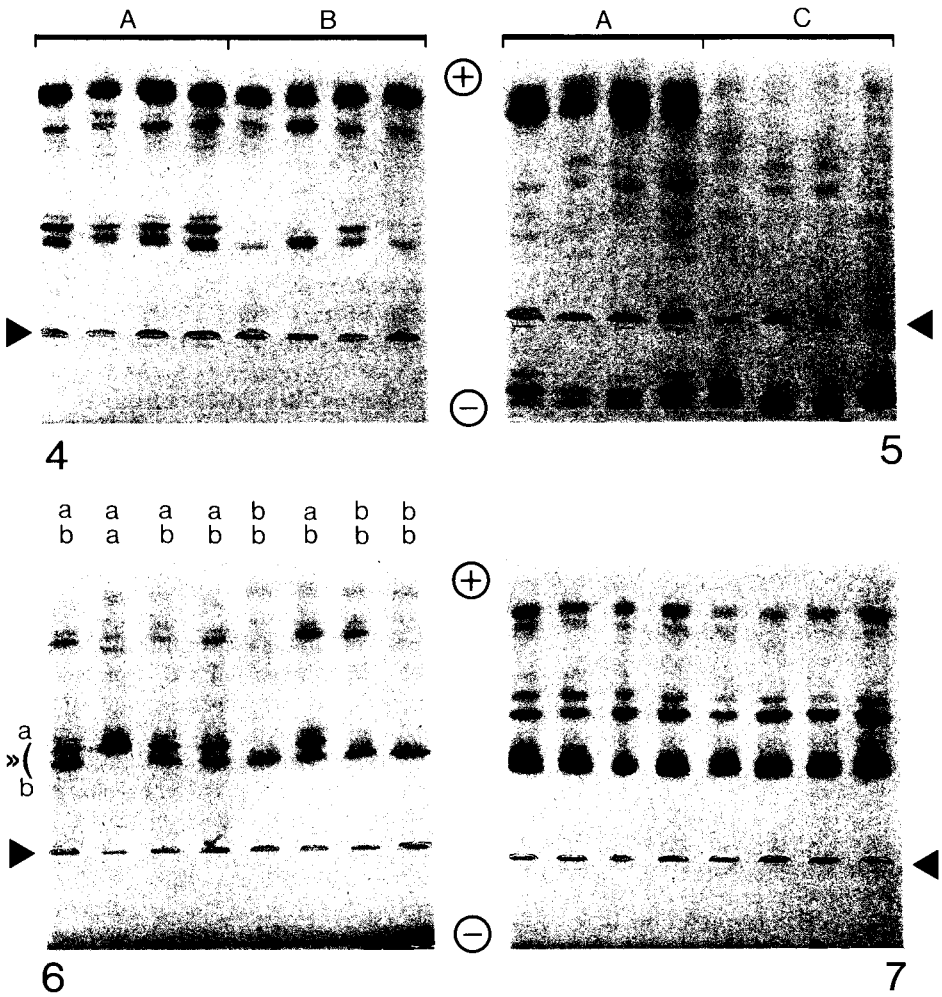
Mill and Grahame (1988) obtained a "reasonable" separation of the morphologically extremely similar *Littorina saxatilis* and *L. arcana* after IEF of non-specific esterases. In addition it was shown that *L. saxatilis* is more variable and heterogeneous than *L. arcana*. Similar results were reported by Dytham *et al.* (1992) and Mill and Grahame (1992), who also observed a clinal change in esterase variation in both periwinkles.

Bivalvia

Günther and Hinz (1986) used IEF of amylases to separate two morphologically similar *Pisidium* species, *viz. P. personatum* and *P. nitidum*. They also noted that two alleles detected by native agarose gel electrophoresis were not resolved by IEF. Yet, subsequently Günther and Hinz (1988) remarked that IEF of amylases was superior to agarose gel electrophoresis in differentiating 15 *Pisidium* and three *Sphaerium* species. The same authors also analyzed phosphoglucomutase with IEF and this, combined with the amylase data, allowed them to confirm: (1) the close relationship between *P. hibernicum* and the group composed of *P. henslowanum*, *P. supinum* and *P. lilljeborgii*, (2) the close relationship between *P. pulchellum* and *P. subtruncatum* and (3) the separate position of *P. amnicum*.

Cephalopoda

Brahma and Lancieri (1979) assessed phylogenetic relationships between *Octopus vulgaris*, *Sepia officinalis*



Figures 4–7. Performance of PhastSystem in a preliminary IEF analysis of esterases in total body homogenates of some *Baicalia* spp. from Lake Baikal. **4.** Interpopulation heterogeneity in *B. costata* (A: Dva Brata; B: Varnachka) (pH 3–9). **5.** Interspecific differentiation between *B. costata* (A: Dva Brata) and *B. turriformis* (C: Dva Brata) (pH 4–6.5). **6.** Genetic variation at a diallelic monomeric esterase in *B. turriformis* (first four specimens from Dva Brata, next four from Varnachka; genotypes are indicated above each lane) (pH 3–9). **7.** Intrapopulation homogeneity of *B. bithyniopsis* (Bolskije Koty) (pH 3–9).

and *Loligo vulgaris* using IEF and immuno-IEF (= IEF followed by an immunodiffusion test against antisera) of eye lens proteins. Immuno-IEF showed a closer relationship between *Sepia* and *Loligo*, than between either of these two and *Octopus*. "Classical" IEF, on the contrary was uninformative as only *Octopus* yielded interpretable IEF profiles.

Lévy *et al.* (1988) used IEF of general proteins of mantle extracts to separate two sibling species of Brazilian *Eledone*, *viz. E. massyae* and *E. gaucha*.

IEF DATA ANALYSIS

The preceding review shows that IEF data have been used in three ways: (1) qualitatively, by seeking taxon specific banding profiles, (2) phenetically, by calculating band pattern similarities and (3) genetically, by interpreting the profiles in terms of loci, alleles and genotypes.

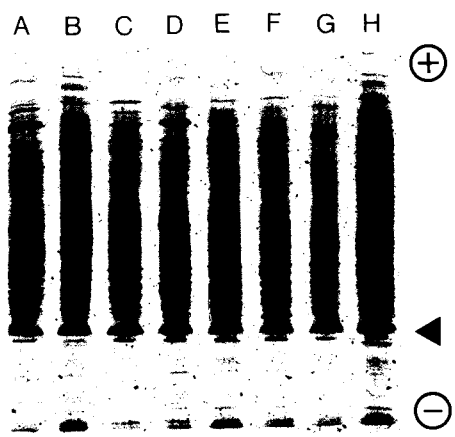
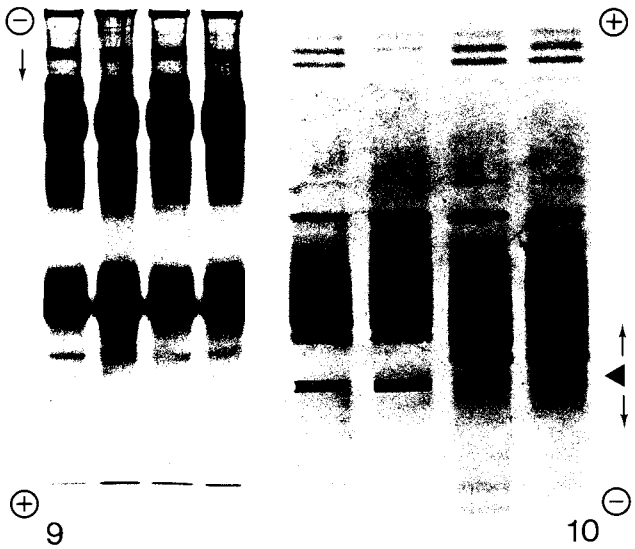


Figure 8. General silver staining of proteins in total body homogenates of *Baicalia ventrosula* (A-D) and *B. herderiana laevis* (E-H) collected at Bolskije Koty (pH 4–6.5).



Figures 9–10. Hidden heterogeneity among albumen gland protein profiles (Coomassie staining) of *Arion hortensis* (Mortsel, Belgium). **9.** Vertical polyacrylamide gel electrophoresis in a 7% gel showing no interindividual variation. **10.** Agarose IEF of the same specimens in a 4–6.5 pH gradient resolving hidden variation in the protein bands near the application site. Migration patterns are indicated by arrows.

Qualitative analyses will not be dealt with further, as they are amply illustrated in our review.

Phenetic analyses are usually performed on banding profiles for which no genetic interpretation is possible (*e.g.*, general protein patterns, uniparental organisms, etc.). Such patterns are compared by the band-counting method (Ferguson, 1980), which treats each band as a distinct character. To this end gels are examined on a light table and adjacent profiles are compared pairwise two under a magnifying lens. The "resemblance" between two profiles can then be expressed by a similarity index, which usually relies on a ratio between shared and unique bands (*e.g.*, Lawson *et al.*, 1980). The simplest index was defined by Ferguson (1980):

$$S_F = \frac{c}{m}$$

where c = number of shared bands and m = maximum number of bands in one of the two compared profiles (*e.g.*, Munuswamy, 1982; Backeljau, 1985; Backeljau *et al.*, 1987; Radice *et al.*, 1988; Verheyen *et al.*, 1991; Phillips *et al.*, 1992).

Three other binary similarity indices have also been used for electrophoretic data. The coefficient of Marczewski and Steinhaus is defined as:

$$S_M = \frac{c}{a_T + b_T - c}$$

where c = number of shared bands, a_T = total number of bands in profile A and b_T = total number of bands in profile B (*e.g.*, Sywula and Bartkowiak, 1978). The matching coefficient of Jaccard (S_J) and its modification (S_{JM}) by Czekanowski [often attributed to Dice or Sörensen (Sneath & Sokal, 1973; Clifford & Stephenson, 1975)] are given by:

$$S_J = \frac{c}{a_U + b_U + c} \qquad S_{JM} = \frac{2c}{a_U + b_U + 2c}$$

where c = number of shared bands, a_U = number of unique bands in profile A and b_U = number of unique bands in profile B. (*e.g.*, Sella & Badino, 1980; Stoddart, 1983; Riutort *et al.*, 1992). S_J has been used by Nixon and Taylor (1977) and Ribas *et al.* (1989) to estimate the time of divergence between noninterbreeding taxa according to Nei's (1971) formula:

$$t = \frac{D}{2cn_T\Omega_a}$$

where $D = -\log_e S_J$, c = the proportion of amino acid substitutions detectable by electrophoresis, n_T = the total number of codons needed to code for a protein and Ω_a = the rate of amino acid substitutions per site per year. However, some assumptions and estimations made by Nei (1971) may not be applicable to IEF profiles of general proteins, because one cannot assign band homologies. Moreover, S_J makes the unrealistic assumption that each band is a unique protein species. Finally, the estimation of c was based on charge characteristics only and thus needs correction in the light of the resolving power of IEF.

The statistical properties of 39 binary similarity indices (including S_J and S_{JM}) have been compared by Shi (1993), who recommended the use of Jaccard's coefficient (S_J), because this index meets most statistical requirements. S_{JM} performs very well too (Shi, 1993), but gives more weight to shared bands (2c). This may be an undesirable property, since electrophoretic data tend to inflate similarity indices due to the fact that shared bands do not necessarily involve identical proteins (hidden heterogeneity). Even though IEF reduces the likelihood of such chance similarities, it does not eliminate them and therefore S_{JM} may be less appropriate. The statistical properties of S_F and S_M have not yet been investigated. Hence their performance relative to S_J is unknown.

If bands can be characterized unambiguously (*e.g.*, by their pI values), one may construct discrete presence/absence data matrices (see also Nixon & Taylor, 1977), which can be subjected to multivariate ordination methods or parsimony programs (*e.g.*, Thorpe, 1985). This latter approach is conceptually similar to the "independent allele" model, which treats each allele as a distinct character with two states (presence/absence). However, the application of this model is highly questionable if not invalid (*e.g.*, Murphy, 1993) and therefore it seems more appropriate to use Gelfand's similarity as an alternative:

$$S_G = \frac{1}{1 + \Sigma (x_i - y_i)^2}$$

where x_i and y_i are the frequencies of the ith band in

populations X and Y, and Σ is taken over the total number of bands in both populations. Thus contrary to S_F, S_M, S_J and S_{JM}, S_G is not applicable to individual comparisons, but to group comparisons (*e.g.*, Cline *et al.*, 1992).

All similarity indices mentioned can be converted in dissimilarities using:

$$DIS = 1 - S$$

A computer program to calculate S_F, S_M and S_G and the corresponding DIS values has been written by Angus *et al.* (1988). S_J and S_{JM} can be calculated with the program NTSYS-pc (Rohlf, 1993). General accounts on similarity indices and their statistical properties can be found in Constandse-Westermann (1972), Sneath and Sokal (1973) and Clifford and Stephenson (1975).

Similarities or distances can be compared hierarchically. With S_F, S_M, S_J and S_{JM}, for example, three levels of relatedness can be considered: (1) intrapopulational, (2) interpopulational and (3) interspecific (*e.g.*, Backeljau, 1985). Average similarities can then be calculated as the arithmetic means of all values for a given class of comparisons. Differences between these means can be tested with an estimation of the standard error of the difference between two means (Farnsworth, 1978), a Student-t-test or an analysis of variance followed by a Duncan Multiple Range test or a Student-Newman-Keuls test (Sokal & Rohlf, 1981). These statistics require that the data are independent, normally distributed and homoscedastic (Sokal & Rohlf, 1981). Deviations from the latter two assumptions can be dealt with by applying data transformations or nonparametric tests (Sokal & Rohlf, 1981; Hageman, 1992). The statistical treatment of interdependent data (*e.g.*, when single profiles contribute to more than one comparison) is a much more fundamental problem, which also applies to similarity values calculated from other molecular data such as Random Amplified Polymorphic DNA (RAPD) profiles (Chapco *et al.*, 1992). So, if mean S_F, S_M, S_J or S_{JM} values are to be tested as outlined above, one should use each individual in only one comparison, such that a set of independent similarity values is generated. Gelfand's index (S_G), on the contrary, can be compared statistically by resampling techniques such as bootstrapping or jackknifing over bands (*e.g.*, Crowley, 1992), followed by an estimation of variances and confidence intervals using, for example, an approach similar to that of Mueller and Ayala (1982).

Finally, phenetic IEF data can yield information with respect to the overall variability of organisms in relation to environmental characteristics. Mill and Grahame (1988, 1992), for example, expressed esterase band heterogeneity among littorinid populations from different sites and species by calculating the Shannon Wiener diversity index for each sample as:

$$DIVERSITY_{SW} = -\Sigma\, p_i \log_e p_i$$

where p_i is the frequency of the ith band in the sample. More generally, in phenetic protein similarity analyses one must always consider possible environmental, developmental and seasonal variations before taxonomic conclusions may be drawn (*e.g.*, Backeljau *et al.*, 1987).

Next to phenetic analyses, IEF data can also be interpreted genetically (*e.g.*, figure 6; Rollinson & Wright, 1984; Théron *et al.*, 1989; Alstad & Corbin, 1990; Rollinson *et al.*, 1990; Alstad *et al.*, 1991). Yet, such approach is not always possible because IEF may occasionally yield genetically uninterpretable profiles produced by artifactual interactions between carrier ampholytes and proteins (Hare *et al.*, 1978; Righetti, 1983).

As the genetic analysis of IEF data proceeds in exactly the same way as for other electrophoretic data, we refer to the extensive literature on these methods for more details (*e.g.*, Richardson *et al.*, 1986; Nei, 1987; Weir, 1990; Whitmore, 1990a; Hillis & Moritz, 1990). Computer packages and programs for various aspects of electrophoretic data analysis have been published by Swofford and Selander (1981), Suiter *et al.* (1983), Swofford and Berlocher (1987), Farris (1989), Lessios (1990), Weir (1990a, b), Felsenstein (1991), Swofford (1991), Lewis (1992) [see also Whitkus, 1985, 1988], Quesada *et al.* (1992) and Ota (1993). This list is not exhaustive. All these programs were written for PC's and larger computers. Yet, there are also programs for Texas Instruments calculators (Spikell & Blumenberg, 1977; Blumenberg & Spikell, 1978, 1980; Blumenberg, 1981).

DISCUSSION

Because of its generally higher resolving power, IEF provides an effective tool to analyse hidden protein variation not detected by conventional electrophoretic methods (Figures 9–10). It is therefore a complementary technique, which is most conveniently used in conjunction with others. An extreme example of this is two-dimensional (2D) electrophoresis. In this approach proteins are separated by IEF in a first dimension and by, for example, SDS electrophoresis in a second dimension perpendicular to the first one. The resulting profiles often show >100 protein spots and thus provide large data sets. Yet, only very few applications of 2D-electrophoresis in molluscan taxonomy have hitherto been published (*e.g.*, Miyazaky *et al.*, 1988; Tsubokawa & Miyazaki, 1993), but both studies clearly show the utility of this method.

Since IEF concentrates proteins on the basis of their isoelectric points it is also a convenient technique to analyse minute organisms (*e.g.*, Kazmer, 1991). Moreover, single IEF runs combined with general protein stainings, often yield considerably larger numbers of discrete characters (bands) than conventional electrophoretic methods. This may be advantageous when only few specimens can be screened (*e.g.*, rare organisms). As such, IEF also provides a means to perform quick preliminary analyses of particular problems (*e.g.*, in order to plan a more extensive survey using other methods). Finally, IEF seems a most efficient technique for species (taxon) identification, particularly since bands can be identified by their pI values and thus can be compared between gels.

Needless to say that IEF can just as well be used for conventional population genetic applications, even though we believe that other electrophoretic methods will continue to dominate this field because of the lower costs involved.

In conclusion, IEF is a technique that has much to offer, particularly when employed in combination with conventional electrophoresis. Nevertheless, its advantageous features are currently far from fully explored or exploited in systematic malacology.

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Appendix 1. Basic guidelines for IEF experimentation.

Sample preparation

Either total body homogenates or specific tissues can be used for IEF. Muscle, mantle, gonad and digestive gland extracts are good sources to resolve specific enzymes, whereas albumen glands, eggs and eye lenses (cephalopods) are more convenient for general protein stainings. Samples have to be prepared on ice to prevent heat denaturation of the proteins. Since salts may distort IEF gradients (Sévigny & Odense, 1985; Robinson, 1989; Whitmore, 1990b), tissues are preferably homogenized in distilled water (*e.g.* Sella & Badino, 1980; Manga-Gonzalez & Rollinson, 1986), to which we add 20% (w/v) sucrose (Backeljau, 1985, 1989). Yet, organic (*e.g.*, Brahma & Lancieri, 1979; Viyanant *et al.*, 1985) or dilute inorganic (*e.g.*, Wright & Rollinson, 1979; Günther & Hinz, 1986, 1988; Mill & Grahame, 1988) buffers are tolerated too. Wright & Rollinson (1979) also added 1.0 mM of dithiothreitol, ϵ-aminocaproic acid and ethylenediamine tetraacetic acid (EDTA) as enzyme stabilizers. Still many more extraction solutions are possible (*e.g.*, Dixon & Arai, 1985; Sévigny & Odense, 1985; Kilias, 1988; Keyvanfar *et al.*, 1988; Holmes *et al.*, 1989; Robinson, 1989; Payan & Dickson, 1990; Phillips *et al.*, 1992).

We add 5 μl extraction solution per mg tissue, but other proportions have been used too: Viyanant *et al.* (1985) and Mill and Grahame (1988) homogenized individual snails in respectively 300 μl and 200 μl buffer; Günther and Hinz (1986, 1988) placed single *Pisidium* specimens in 50 μl solution; Wright and Rollinson (1979) and Sella and Badino (1980) used 1:1 proportions, while Brahma and Lancieri (1979) prepared 2% (w/v) homogenates. Tissues may be homogenized with a pestle and mortar (Mill & Grahame, 1988), a mixer (Sella & Badino, 1980; Backeljau, 1989) or a sonicator (Günther & Hinz, 1986, 1988). Viyanant *et al.* (1985) first homogenized snails with a mixer and subsequently sonicated the suspensions three times for 20 sec at 150 W. Homogenates are subsequently centrifuged during 30–45 min at 18000 × g (= 13000 r.p.m.) to 27000 × g (= 15000 r.p.m.) (4°C) (Backeljau, 1985, 1989). Following regimes have also been reported: 25 min at 50000 × g (Wright & Rollinson, 1979), 10 min at 6000 r.p.m. and 5 min at 12000 r.p.m. (Sella & Badino, 1980), 30 min at 12000 r.p.m. (Viyanant *et al.*, 1985) and 4 min at 5000 r.p.m. (Herberts *et al.*, 1989). Brahma and Lancieri (1979) used glass fiber papers (5 × 10 mm) to absorb 20 μl extract without centrifugation. Mill and Grahame (1988) centrifuged their homogenates during 2.5 min at 4000 r.p.m. and freeze-dried the supernates. These were rehydrated with distilled water when needed.

Supernates can be stored at or below −70°C. Some proteins, however, may denature at these temperatures

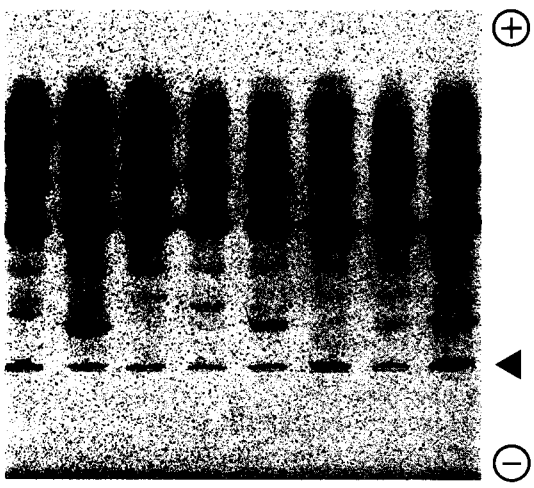

Figure 11. IEF (pH gradient 3–9) patterns of esterases from digestive gland homogenates of *Bukobia* sp. (Mufindi, Tanzania) after more than five years of storage at –70°C. IEF was performed with PhastSystem. Note the granulation caused by undissolved Fast Blue RR (cf. figures 4–7).

(Sévigny & Odense, 1985; Privalov, 1990). For long term storage, it is better to freeze complete individuals or tissues, than to store extracts. Nevertheless, albumen gland homogenates of arionids did not deteriorate over a period of six years, even after repeated freezing and thawing (Backeljau, 1989). Similarly, complete *Bukobia* specimens, stored for more than five years at –70°C, still yielded satisfactory and reproducible esterase profiles (figure 11). The need of fresh material and its storage are disadvantages of protein electrophoresis in general. However, Westheide and Brockmeyer (1992) published protocols for IEF of ethanol-fixed oligochaetes. Taylor *et al.* (1994) reported the possibility of air-drying samples in 15% (w/v) trehalose.

Table 1. Programmed conditions for IEF separations in two pH gradients in mini polyacrylamide gels using PhastSystem (tested with esterases and albumen gland proteins).

		pH 3–9			
Sample appl. down at		2.2			0 Vh
Sample appl. up at		2.3			0 Vh
Extra alarm sound at		2.1			73 Vh
SEP 2.1	2,000 V	2.5 mA	3.5 W	5 °C	75 Vh
SEP 2.2	200 V	2.5 mA	3.5 W	5 °C	15 Vh
SEP 2.3	2,000 V	2.5 mA	3.5 W	5 °C	510 Vh
		pH 4–6.5			
Sample appl. down at		1.2			0 Vh
Sample appl. up at		1.3			0 Vh
Extra alarm sound at		1.1			73 Vh
SEP 1.1	2,000 V	2.0 mA	3.5 W	5 °C	75 Vh
SEP 1.2	200 V	2.0 mA	3.5 W	5 °C	15 Vh
SEP 1.3	1,500 V	4.0 mA	3.5 W	5 °C	510 Vh

Table 2. Programmed silver staining procedure as used with the PhastSystem development unit. EtOH = ethanol, HAc = Acetic acid, TCA = Trichloroacetic acid. Background reducer consists of 2.5 g sodium thiosulphate + 3.7 g Tris in 10 ml reagent grade water; developer consists of 1 ml 2% formaldehyde + 150 ml 2.5% sodium carbonate.

Dev	Solution	In	Out	Time (min)	T (°C)
1	20% TCA	1	0	5	20
2	10% EtOH 5% HAc	3	0	2	50
3	10% EtOH 5% HAc	3	0	4	50
4	5% Glutaraldehyde	4	0	6	50
5	10% EtOH 5% HAc	3	0	3	50
6	10% EtOH 5% HAc	3	0	5	50
7	reagent grade H_2O	5	0	2	50
8	reagent grade H_2O	5	0	2	50
9	0.4% $AgNO_3$	6	0	10	40
10	reagent grade H_2O	5	0	0.5	30
11	reagent grade H_2O	5	0	0.5	30
12	developer	7	0	0.5	30
13	developer	7	0	3.5	30
14	background reducer	8	0	1.5	30
15	reagent grade H_2O	5	0	5	50

Casting and running IEF gels

IEF is usually performed in polyacrylamide (PAA) or agarose gels. Information on PAA gel preparation is provided by Righetti (1983), Viyanant and Upatham (1985), Nunamaker and McKinnon (1989), Robinson (1989), Mork (1990), Whitmore (1990b) and Westheide and Brockmeyer (1992). Agarose recipes can be found in Righetti (1983), Sévigny and Odense (1985), Whitmore (1986), Backeljau (1989) and Dixon and Arai (1989). Most of these references also provide protocols for IEF running conditions. Additional information can be found in Righetti *et al.* (1990) and Whitmore (1990a).

Recently, LKB-Pharmacia introduced an automated electrophoretic unit (PhastSystem) capable of executing, among others, horizontal IEF in mini PAA gels of 50 × 43 × 0.35 mm (Olsson *et al.*, 1988a). In this unit all running conditions are controlled by a programmable microprocessor. It achieves exactly the same resolution as 'manual' IEF in larger gels, but in much shorter times (± 30 min). Table 1 lists our PhastSystem programs for IEF separations of esterases and general proteins in two pH gradients, while figures 4–7 and 11 illustrate some separations obtained with these programs.

Gel staining

After IEF, gels can be stained for either nonspecific proteins or specific enzymes. Recipes for the latter are essentially the same as those published for conventional electrophoresis (*e.g.* Harris & Hopkinson, 1976; Richardson *et al.*, 1986; Morizot & Schmidt, 1990; Murphy *et al.* 1990). Righetti (1983) provided a review of stainings applied in IEF. Some recipes used for molluscs are

given by Wright and Rollinson (1979) and Manga-Gonzalez and Rollinson (1986).

Our recipe for esterase staining is as follows (Backeljau, 1985): dissolve 40 mg Fast Blue RR in a mixture of 25 ml 0.1M KH_2PO_4/NaOH buffer at pH 7.0 (= 6.804 g KH_2PO_4 + 1.164 g NaOH in 1000 ml H_2O), 25 ml H_2O and 2 ml α-naphtylacetate solution (1% w/v α-naphtylacetate in 50% v/v acetone). Before pouring this solution on the gel, it should be filtered to avoid precipitation of undissolved Fast Blue RR (figure 11). Staining takes about 45 min.

General proteins are often stained with Coomassie Brilliant Blue R-250 (= Serva Blue R), as outlined by Backeljau (1989). Silver staining, however, is more sensitive (*e.g.*, Rabilloud, 1990). Several recipes are provided by Righetti (1983). The programmed protocol we follow with PhastSystem (Olsson *et al.* 1988b) is given in table 2.

Agarose and thin PAA gels can be dried and stored after staining. PhastSystem gels can be kept as slides. After prolonged storage (> two years) gels stained for esterases may be covered by a white "dust". This can be washed away by rinsing the gel under gently running tap water. Specific enzyme stainings are less stable for long term storage. Therefore we recommend to photograph or photocopy all gels.

Appendix 2. Systematic list of the molluscan taxa mentioned.

CLASS: GASTROPODA

Subclass: Prosobranchia

Fam. Patellidae
Patella aspera Röding, 1798
Patella coerulea Linnaeus, 1758
Patella lusitanica Gmelin, 1791

Fam. Bithyniidae
Bithynia funiculata Walker, 1927
Bithynia siamensis siamensis Lea, 1856
Bithynia siamensis goniomphalos (Morelet, 1866)

Fam. Baicaliidae
Baicalia (Baicalia) turriformis Dybowski, 1875
Baicalia (Maackia) costata Dybowski, 1875
Baicalia (Eubaicalia) bithyniopsis Lindholm, 1909
Baicalia (Eubaicalia) herderiana laevis Kozhov, 1936
Baicalia (Eubaicalia) ventrosula Lindholm, 1909

Fam. Ampullariidae (= Pilidae)
Pomacea canaliculata (Lamarck, 1804)

Fam. Littorinidae
Littorina saxatilis (Olivi, 1792)
Littorina arcana Hannaford-Ellis, 1978

Subclass: Heterobranchia (partim Pulmonata)

Fam. Planorbidae
Bulinus liratus (Tristram, 1863)
Bulinus obtusispira (Smith, 1882)
Bulinus africanus (Krauss, 1848)
Bulinus nasutus (von Martens, 1879)
Bulinus senegalensis Müller, 1781
Bulinus tropicus (Krauss, 1848)
Bulinus truncatus (Audouin, 1827)
Bulinus coulboisi (Bourguignat, 1888)
Bulinus guernei (Dautzenberg, 1890)
Bulinus cernicus (Morelet, 1867)
Bulinus permembranaceus (Preston, 1912)

Fam. Arionidae
Arion (Kobeltia) hortensis Férussac, 1819
Arion (Kobeltia) distinctus Mabille, 1868
Arion (Kobeltia) owenii Davies, 1979
Arion (Kobeltia) fagophilus de Winter, 1986
Arion (Kobeltia) intermedius Normand, 1852
Arion (Carinarion) fasciatus (Nilsson, 1823)
Arion (Carinarion) circumscriptus Johnston, 1828
Arion (Carinarion) silvaticus Lohmander, 1937

Fam. Urocyclidae
Bukobia sp.

Fam. Helicidae
Helicella sp.

CLASS: BIVALVIA

Fam. Sphaeriidae
Pisidium personatum Malm, 1855
Pisidium nitidum Jenyns, 1832
Pisidium hibernicum Westerlund, 1894
Pisidium henslowanum (Sheppard, 1823)
Pisidium supinum Schmidt, 1851
Pisidium lilljeborgii Clessin, 1886
Pisidium pulchellum Jenyns, 1832
Pisidium subtruncatum Malm, 1855
Pisidium amnicum (Müller, 1774)
Sphaerium sp.

CLASS: CEPHALOPODA

Fam. Sepiidae
Sepia officinalis Linnaeus, 1758

Fam. Loliginidae
Loligo vulgaris Lamarck, 1798

Fam. Octopodidae
Octopus vulgaris Cuvier, 1798
Eledone massyae Voss, 1964
Eledone gaucha Haimovici, 1988