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Isoelectric focusing: A rapid method for identification and differentiation of teleost species

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ABSTRACT Isoelectric focusing (IEF) of sarcoplasmic proteins was conducted using Ampholine® PAGplates (Pharmacia, Sweden) of pH 3.5-9.5 to examine and differentiate various teleost species. Different genera and species within five teleost families, i.e., Belontiidae (*Betta splendens*, *B. pugnax*, *Colisa lalia*, *Trichogaster leeri* and *T. trichopterus*), Channidae (*Channa striata* and *C. micropeltes*), Cichlidae (*Oreochromis mossambicus*, *O. niloticus*, *O. aureus*, *Astronotus ocellatus* and *Pterophyllum scalare*), Cyprinidae (*Barbus tetrazona*, *Carassius auratus*, *Cyprinus carpio* and *Leptobarbus hoeveni*) and Poeciliidae (*Poecilia velifera*, *P. latipinna*, *P. sphenops*, *P. reticulata*, *Xiphophorus helleri* and *X. maculatus*) were analysed to detect variations in their sarcoplasmic protein patterns. Comparisons of IEF profiles were made among conspecifics, species within a genus, genera within each family and among families. Our findings showed that IEF could (a) produce unique species-, genus- and family-specific patterns resulting from inherent genetic factors, and (b) determine the extent of protein polymorphisms due to genetic variations among individuals from pure strains and hybrids. The applicability of IEF as a simple and reliable technique with excellent resolution for rapid preliminary examination of fishes at taxonomic levels and fisheries management is also discussed.

ABSTRAK Penumpuan isoelektrik (IEF) protein sarko-plasma telah dilakukan dengan menggunakan kepingan PAG Ampholine® (Pharmacia, Sweden) pada pH 3.5-9.5 untuk memeriksa dan membezakan berbagai spesies ikan teleost. Spesies-spesies daripada famili Belontiidae (*Betta splendens*, *B. pugnax*, *Colisa lalia*, *Trichogaster leeri* dan *T. trichopterus*), famili Channidae (*Channa striata* dan *C. micropeltes*), famili Cichlidae (*Oreochromis mossambicus*, *O. niloticus*, *O. aureus*, *Astronotus ocellatus* dan *Pterophyllum scalare*), famili Cyprinidae (*Barbus tetrazona*, *Carassius auratus*, *Cyprinus carpio* dan *Leptobarbus hoeveni*) dan famili Poeciliidae (*Poecilia velifera*, *P. latipinna*, *P. sphenops*, *P. reticulata*, *Xiphophorus helleri* dan *X. maculatus*) telah dianalisa untuk mengesan variasi corak protein sarkoplasma mereka. Perbandingan oleh profil IEF telah dilakukan terhadap individu-individu dalam setiap spesies, spesies-spesies dalam setiap genus, genus-genus dalam setiap famili dan antara setiap famili ikan yang dikaji. Penemuan kami mendapati bahawa IEF boleh (a) menentukan keunikan spesies, genus dan juga famili daripada aspek genetik, dan (b) menentukan tahap polimorfisme protein yang disebabkan oleh variasi genetik di antara individu-individu dari strain tulen dan hibrid.

(Isoelectric focusing, sarcoplasmic proteins, teleosts)

INTRODUCTION

Electrophoresis has been used extensively for the identification and classification of morphologically similar and closely related teleost species, for example, coregonines [1], tilapiines [2,3] and rainbow trout [4]. Among polyacrylamide gel electrophoresis (PAGE) techniques, isoelectric focusing (IEF) has emerged as a powerful tool for routine species identification of tissues from foodfishes [5,6,7]. Using IEF, only a single specimen is required to establish a species, and results are reproducible with minor variations due to genetic polymorphism in samples from different geographic locations and populations. The value of IEF is evident in studies for which morphological data are insufficient. This was shown by Ng *et al.* [7] in differentiating the threadfin bream, *Nemipterus* species, and Ng [8] in distinguishing the piranha, *Serrasalmus nattereri*, from the closely resembling pacu, *Colossoma brachypomum.*

In conventional PAGE, protein migration through a gel of a given pH is dependent on molecular weight. IEF, on the other hand, uses an equilibrium technique to resolve proteins according to their isoelectric points (pIs) in a pH gradient formed by carrier ampholytes [5,6,7]. Proteins migrate in an electric field along this stabilised pH gradient until they reach a pH equivalent to their isoelectric points where they then lose their charges and become concentrated into very sharp bands. Polymorphic (variable) bands resulting from variations in enzymes (allozymes and isozymes) and proteins are considered as genetic markers. The detection of genetic markers is of vital importance for stock management in aquacultural programmes and natural populations. These polymorphic markers have been widely and successfully utilised in population genetic studies of the black seabream, *Acanthopagrus schlegeli* [9,10], tilapia, *Oreochromis mossambicus* and *O. niloticus* [11,12],

rosy threadfin bream, *Nemipterus peronii* [13], Atlantic cod, *Gadus morhua* [14], Siamese fighting fish, *Betta splendens* [15] and the platyfish, *Xiphophorus maculatus* [16].

To date, the use of IEF has largely been concentrated on marine and temperate water foodfish species that are of economic importance in aquaculture. Application of IEF to ornamental and freshwater species is, however, still in its infancy. In this study, IEF was used to resolve the sarcoplasmic proteins of 22 species of tropical fishes representing 13 genera in five families of the Teleostei, *viz.*, Belontiidae (*Betta splendens*, *B. pugnax*, *Colisa lalia*, *Trichogaster leeri*, *T. trichopterus*), Channidae (*Channa striata*, *C. micropeltes*), Cichlidae (*O. mossambicus*, *O. niloticus*, *O. aureus*, *Astronotus ocellatus*, *Pterophyllum scalare*), Cyprinidae (*Barbus tetrazona*, *Carassius auratus*, *Cyprinus carpio*, *Leptobarbus hoeveni*) and Poeciliidae (*Poecilia velifera*, *P. latipinna*, *P. sphenops*, *P. reticulata*, *Xiphophorus helleri*, *X. maculatus*). We report here the applicability of IEF in taxonomy, systematics and stock management of cultured ornamental and freshwater fish species.

MATERIALS AND METHODS

Sarcoplasmic proteins were extracted according to Khoo [15] from four individuals of each species (three for poeciliids) by homogenising approximately 1:5 (w/v) muscle tissue from the antero-dorsal region of the fish in cold (5-10°C) distilled water with a chilled Eppendorf micropestle (Germany). The homogenate was centrifuged at 8,000 rpm for 20 min (4°C) with a Beckman J2-21 centrifuge and JA18.1 rotor (USA) to pellet cell debris. Protein samples (clear supernatant) were collected and stored at -85°C until required.

The IEF procedure of Khoo [15], modified from the protocols of Lundstrom and Roderick [5] and Ng *et al.* [7], was used. Isoelectric focusing was conducted in an LKB Multiphor II Electrophoresis Unit (Pharmacia, Sweden) equipped with a Lauda MGW RM-20 thermostatic recirculation unit (Sweden). Thawed samples were applied onto commercially pre-cast gels (Ampholine® PAGplate, pH 3.50-9.30) at 15 µl/application piece. To establish pI values following IEF, the Pharmacia Broad pI Calibration Kit (pH 3-10) with 11 purified protein standards and a methyl red marker dye ranging from pI 3.50 to 9.30 was used. Anode and cathode electrode strips were evenly soaked with 1 M H_3PO_4 and 1 M NaOH, respectively. All gels were run at 1,300 V, 50 mA and 30 W for 100 min (4(C) with a Pharmacia Multiphor Multidrive XL power supply.

After electrofocusing, gels were fixed in a solution of trichloroacetic acid [TCA] and sulphosalicylic acid [SSA] (29 g TCA and 8.5 g SSA in 250 mL distilled H_2O) for 60 min. They were then rinsed thrice with a 25% ethanol – 8% acetic acid destaining solution and stained for 8-10 min with filtered pre-heated (60°C) Coomassie Blue R-250 solution (0.29 g Coomassie Brilliant Blue R-250 in 250 mL destaining solution). Gels were destained by renewing the destaining solution until their background was colourless. After destaining, the gels were placed in a preserving solution (10% glycerol in 250 mL destaining solution) for 60 min and covered with cellophane (soaked in preserving solution for over 30 min). Mounted gels were dried overnight at 50°C in a Memmert ventilated incubator (Germany). All chemicals for staining, unless otherwise stated, were from Sigma, USA.

Dried PAG plates were photographed on a light-table using a Nikon FG camera fitted with a 55 mm f2.8 Micro-Nikkor lens and Hoya R(25A) infra-red filter (Japan). Kodak TMAX 100 black and white negative film was used. Densitometric readings were generated using the Bio-Gene V5.04 software (Vilber Lourmat, France) after images of PAG plates were scanned automatically with an attached CCD camera. The pI values were determined by comparing the distance from the cathodic end to the peak of each protein band in the samples with those in the protein standards. To measure genetic similarity and variability, pairwise comparisons of individuals were made within and between species in each teleost family. Band Sharing Index (BSI) was calculated using the Bio-Gene software according to the following formula, $BSI = 2N_{AB}/(N_A+N_B)$, where N_{AB} is the number of bands shared in common by individuals A and B, and N_A and N_B the total number of bands for A and B, respectively. BSI values may range from 0 when there are no bands shared between the IEF patterns of two individuals, to 1 when no differences are observed in the profiles, i.e., they are identical.

RESULTS AND DISCUSSION

The sarcoplasmic proteins of 22 species comprising 13

genera of tropical fishes were resolved in the present study using IEF. Over the whole effective range of the gels (pH 3.5-9.3), more than 30 distinguishable bands were detected for each species. In all five families, major bands were located in the range of pH 3.5 to 7.3 (Figs. 1A-E). Alkaline bands from pI 7.4 to 9.3 were noted for specimens in *Channa*, *Oreochromis*, *Astronotus*, *Pterophyllum*, *Barbus*, *Carassius*, *Cyprinus*, *Leptobarbus*, *Poecilia* and *Xiphophorus*. IEF patterns in both acidic and alkaline regions of the gels should be used for species differentiation. As shown in Figs. 1A-E, each species exhibited its own distinctive electrophoretic pattern. IEF could also easily distinguish species within the same genus, e.g., *Betta* (*B. splendens* and *B. pugnax*), *Trichogaster* (*T. leeri* and *T. trichopterus*), *Channa* (*C. striata* and *C. micropeltes*), *Oreochromis* (*O. mossambicus*, *O. niloticus* and *O. aureus*), *Poecilia* (*P. velifera*, *P. latipinna*, *P. sphenops* and *P. reticulata*), and *Xiphophorus* (*X. helleri* and *X. maculatus*). Each genus was characterised by a number of shared bands across the species examined. Intergeneric variations in the IEF profiles were, as expected, visually distinct. Each family also revealed unique sarcoplasmic patterns (Figs. 1A-E). Based on these observations, our results support the use of muscle proteins to ascertain systematic relatedness among fish species. Additionally, sarcoplasmic proteins are preferable to other non-enzymatic proteins such as transferrins, serum proteins, haemoglobins, albumins and crystallins for electrophoresis, because they are usually monomorphic (consistent) within a species or population, and are markedly more conservative [17].

Table 1(A-E) shows the BSI values in matrices for all the species examined. The highest value of within species similarity was obtained for the selectively inbred red strain of *A. ocellatus* (BSI = 0.973 ± 0.029) and the lowest for *T. leeri* (BSI = 0.848 ± 0.144). Comparisons within each species showed high BSI values (> 0.84), thus denoting high genetic similarity. As for between species analyses, BSI was highest for the comparison between *O. niloticus* and *O. aureus* (0.744 ± 0.055) but lowest for *B. splendens* and *T. leeri* (0.524 ± 0.040). Cichlid species showed a greater proportion of shared banding patterns (BSI > 0.6) compared to species in the other families (compare Fig. 1C & Table 1C with A, B, D & E). For comparisons between species from different families, we consistently obtained low BSI values of 0.30 to 0.49 (data not shown). We found that IEF could differentiate strains and hybrids of a species through variations in their protein structure and multiple forms of polymorphic proteins that lead to the absence or presence, and varying intensity of certain bands. Figs. 1A, C & D show this capability in the comparisons between the Dwarf and Sunset gouramies, *C. lalia* (BSI = 0.759 ± 0.039), Tiger and Red oscars, *A. ocellatus* (BSI = 0.908 ± 0.044), wild-type and Moss Green tiger barbs, *B. tetrazona* (BSI = 0.743 ± 0.047) and veiltail and fantail goldfish, *C. auratus* (BSI = 0.726 ± 0.031) (Table 1A, C & D).

In this preliminary study, we used only a few specimens (three for poeciliids and four for the other fish species). To provide sufficient evidence for constructing taxonomic and systematic relationships among teleost species, IEF data should be generated using an adequate sample size (> 5) for each species. This will allow more sensitive detection of protein polymorphism and permit additional confirmation of species identity. We demonstrate the latter application in specimens identified by morphometric characters to be presumably the Nile tilapia, *O. niloticus* and sailfin molly, *P. latipinna*. Specimens of these two species, however, revealed very inconsistent IEF patterns (Fig. 1C & E). Two individuals identified as *O. niloticus* seemed to be a hybrid of *O. mossambicus* while the other two had almost similar profiles to *O. aureus*. This may account for the comparatively high BSI value of 0.744 ± 0.055 for *O. niloticus* and *O. aureus*. The three sailfin mollies (silver variant) examined were sold commercially as *P. latipinna* and similarly identified by their 14-15 dorsal fin rays [18]. Two specimens, however, showed similar IEF patterns as *P. velifera* (number of dorsal fin rays = 18-19), and the remaining one might have been either a *P. velifera* hybrid or a true *P. latipinna*.

Species-specific protein markers are invaluable for species verification and the establishment of the true status of certain teleost species for which there is controversy. In many instances, species definition is unsuccessful or contentious due to the lack of differences in morphometric and meristic characters. Alternatively, diversity in coloration and behavioural patterns have been used by taxonomists to distinguish species, e.g., within the Old World Asian knifefish family of Notopteridae (*Chitala* and *Notopterus*) [19]. With the discovery of new and rare species, there arises a need to revise the systematics of many genera, for in-

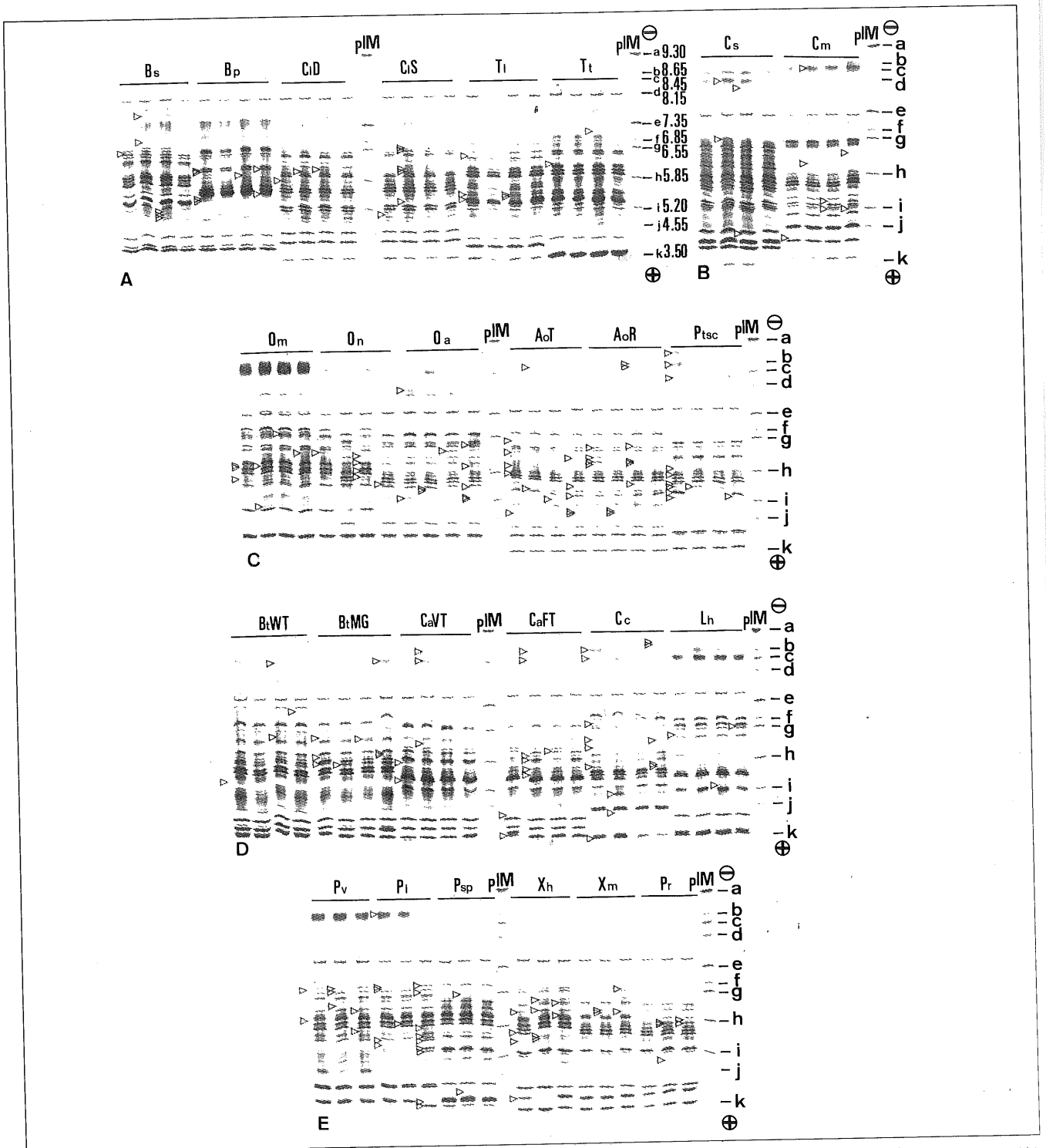

Figure 1. Isoelectric focusing patterns of sarcoplasmic proteins showing species-, genus- and family-specific profiles within Family **(A) Belontiidae** (Bs: *Betta splendens*, Bp: *B. pugnax*, Cl: *Colisa lalia* [D Dwarf, S Sunset], Tl: *Trichogaster leeri*, Tt: *T. trichopterus*); **(B) Channidae** (Cs: *Channa striata*, Cm: *C. micropeltes*); **(C) Cichlidae** (Om: *Oreochromis mossambicus*, On: *O. niloticus*, Oa: *O. aureus*, Ao: *Astronotus ocellatus* [T Tiger, R Red], Ptsc: *Pterophyllum scalare*); **(D) Cyprinidae** (Bt: *Barbus tetrazona* [WT wild-type, MG Moss Green], Ca: *Carassius auratus* [VT veiltail, FT fantail], Cc: *Cyprinus carpio*, Lh: *Leptobarbus hoeveni*) and **(E) Poeciliidae** (Pv: *Poecilia velifera*, Pl: *P. latipinna*, Psp: *P. sphenops*, Xh: *Xiphophorus helleri*, Xm: *X. maculatus*, Pr: *P. reticulata*). Lanes pIM: Pharmacia broad range pI standards (a: 9.30, b: 8.65, c: 8.45, d: 8.15, e: 7.35, f: 6.85, g: 6.55, h: 5.85, i: 5.20, j: 4.55, k: 3.50). Protein polymorphisms due to genetic variation among individuals of each species are shown as open arrowheads. (Cathode end denoted by ' – ' and anode ' + ').

Table 1. Band Sharing Indices (BSI, Mean ± SD) generated using the Bio-Gene software for species in five teleost families. The leading diagonal gives values for within species pairwise comparisons of individuals while other values denote comparisons between species for Family **(A) Belontiidae** (*B. s: Betta splendens, B. p: B. pugnax,* C. l: *Colisa lalia* [D Dwarf, S Sunset], *T. l: Trichogaster leeri, T. t: T. trichopterus*); **(B) Channidae** (*C. s: Channa striata, C. m: C. micropeltes*); **(C) Cichlidae** (*O. m: Oreochromis mossambicus, O. n: O. niloticus, O. a: O. aureus, A. o: Astronotus ocellatus* [T Tiger, R Red], *Pt. sc: Pterophyllum scalare*); **(D) Cyprinidae** (*B. t: Barbus tetrazona* [WT wild-type, MG Moss Green], *C. a: Carassius auratus* [VT veiltail, FT fantail], *C. c: Cyprinus carpio, L. h: Leptobarbus hoeveni*) and **(E) Poeciliidae** (*P. v: Poecilia velifera, P. l: P. latipinna, P. sp: P. sphenops, X. h: Xiphophorus helleri, X. m: X. maculatus, P. r: P. reticulata*).

(A) Belontiidae

B. s	*B. p*	*C. l* (D)	*C. l* (S)	*T. l*	*T. t*	
0.870 ±0.121	0.740 ±0.062	0.578 ±0.048	0.626 ±0.048	0.524 ±0.040	0.594 ±0.054	*B. s*
	0.905 ±0.088	0.681 ±0.058	0.668 ±0.039	0.562 ±0.038	0.603 ±0.038	*B. p*
		0.912 ±0.092	0.759 ±0.039	0.588 ±0.063	0.559 ±0.062	*C. l* (D)
			0.914 ±0.080	0.538 ±0.055	0.566 ±0.049	*C. l* (S)
				0.848 ±0.144	0.682 ±0.040	*T. l*
					0.909 ±0.084	*T. t*

(B) Channidae

C. s	*C. m*	
0.924 ±0.071	0.574 ±0.044	*C. s*
	0.916 ±0.074	*C. m*

(C) Cichlidae

O. m	*O. n*	*O. a*	*A. o* (T)	*A. o* (R)	*Pt. sc*	
0.938 ±0.063	0.664 ±0.055	0.611 ±0.033	0.623 ±0.040	0.659 ±0.035	0.606 ±0.023	*O. m*
	0.878 ±0.109	0.744 ±0.055	0.683 ±0.045	0.715 ±0.043	0.604 ±0.046	*O. n*
		0.937 ±0.059	0.701 ±0.030	0.728 ±0.023	0.647 ±0.027	*O. a*
			0.946 ±0.053	0.908 ±0.044	0.693 ±0.045	*A. o* (T)
				0.973 ±0.029	0.703 ±0.050	*A. o* (R)
					0.913 ±0.082	*Pt. sc*

(D) Cyprinidae

B. t (WT)	*B. t* (MG)	*C. a* (VT)	*C. a* (FT)	*C. c* (K)	*L. h*	
0.887 ±0.100	0.743 ±0.047	0.613 ±0.049	0.556 ±0.034	0.627 ±0.056	0.673 ±0.059	*B. t* (WT)
	0.925 ±0.068	0.624 ±0.044	0.543 ±0.030	0.639 ±0.045	0.651 ±0.058	*B. t* (MG)
		0.865 ±0.119	0.726 ±0.031	0.581 ±0.050	0.618 ±0.055	*C. a* (VT)
			0.928 ±0.069	0.632 ±0.047	0.541 ±0.037	*C. a* (FT)
				0.904 ±0.108	0.611 ±0.025	*C. c* (K)
					0.882 ±0.126	*L. h*

(E) Poeciliidae

P. v	*P. l*	*P. sp*	*X. h*	*X. m*	*P. r*	
0.892 ±0.129	0.741 ±0.081	0.627 ±0.035	0.528 ±0.029	0.594 ±0.027	0.542 ±0.056	*P. v*
	0.867 ±0.163	0.633 ±0.027	0.527 ±0.024	0.620 ±0.046	0.617 ±0.065	*P. l*
		0.935 ±0.081	0.599 ±0.030	0.570 ±0.011	0.598 ±0.044	*P. sp*
			0.945 ±0.069	0.661 ±0.033	0.581 ±0.021	*X. h*
				0.927 ±0.084	0.676 ±0.025	*X. m*
					0.913 ±0.096	*P. r*

stance, the Southeast Asian giant gouramy genus *Osphronemus* [20], the highly speciose fighting fish genus *Betta* [21] and the snakehead genus *Channa* [22]. Since many of these species are meristically identical and uniform, or have morphologically overlapping characters, we recommend the usage of IEF together with existing taxonomic methods to clarify the status of these species. Also, we suggest the setting up of a reference collection or photographic library of IEF patterns specifically for taxonomic identification. Protein polymorphism in samples can be overcome by identifying the different polymorphic forms and including these patterns in the reference collection. To screen for existing polymorphisms, morphometrically similar species from various geographic locations and populations must be examined. This collection will facilitate side-by-side reference for species identified, and presumably authenticated, by conventional taxonomic techniques.

The ornamental fish industry encompasses a wide range of species marketed throughout the world. Geographic distribution, race variations and other genetic adaptations within a species or population lead to changes in protein structure. In different fish populations, genetic variations result in the expression of protein polymorphisms that are easily detectable by IEF. Figs. 1A-E show some of the unique polymorphisms that occur among individuals within each fish species. These genetic markers located at different polymorphic loci have been used extensively in fisheries management to establish the population structure of a species and the effect of isolation [17], assess the levels of genetic variability and inbreeding in stocks from different geographic locations and populations [9,10,14], study the extent of introgressive hybridisation between cultured species [11,12] and analyse the genetic differences in phenotypic mutants or strains [4,15,16]. These studies are necessary for the maintenance of genetic diversity, enhancement of performance of young in hatcheries and genetic improvement of stocks through selective breeding and hybridisation programmes.

In conclusion, our results show that IEF can be utilised to generate sarcoplasmic protein patterns or fingerprints that are characteristic of each fish species. IEF is able to provide a simple, rapid and reliable tool to complement conventional methods of taxonomy and systematics for the identification and differentiation of fish species. Furthermore, it can be adapted to give a useful estimate of genetic variability, inbreeding levels and phylogenetic relatedness, and is applicable for other protein marker-based studies of commercially valuable cultured ornamental and freshwater species.

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