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RADULAR MYOGLOBIN AS A MOLECULAR MARKER IN LITTORINID SYSTEMATICS (CAENOGASTROPODA)

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ABSTRACT Radular myoglobin (Mb) was investigated in 288 specimens of 10 littorinid species using vertical polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF). Within the genus *Littorina* the two most basal species, *L. striata* and *L. keenae*, have Mb patterns that correspond to those of the genera *Littoraria* and *Nodilittorina*, while the sibling species *L. scutulata* and *L. plena* have identical Mb profiles that consistently differ from those of *L. littorea*, *L. saxatilis*, *L. compressa* and *L. arcana*. In contrast to previous claims, Mb does not consistently separate the sibling rough periwinkles *Littorina saxatilis* and *L. arcana*. These data suggest (1) that the *Nodilittorina/Littoraria* Mb profile in *L. striata* is not unique within the genus *Littorina* and therefore does not refute the assignment of *L. striata* to this genus, and (2) that *L. scutulata* and *L. plena* occupy a separate position compared to the other species of the subgenus *Littorina*. This latter result supports the suggestion that *L. scutulata* and *L. plena* may constitute a separate subgeneric taxon. Finally, the IEF Mb profiles of *Nodilittorina hawaiiensis* and *Cenchritis muricatus* were nearly identical to the *Nodilittorina/Littoraria* Mb pattern. Yet, PAGE of Mb in *Cenchritis muricatus* suggests a tentative Mendelian polymorphism. It is concluded that littorinid Mb may not be a useful marker to distinguish closely related species, but rather provides information on 'higher level' systematics.

KEY WORDS: Caenogastropoda, isoelectric focusing, Littorinidae, myoglobin, protein electrophoresis, systematics

INTRODUCTION

With only two published studies prior to 1998, radular myoglobin (Mb) has not been widely used in littorinid systematics and population genetics (Wium-Andersen 1970, Jones 1972). This is not unexpected since the genetic background of Mb variation in periwinkles remains obscure and controversial (Olabarria et al. 1998). Nevertheless, it has been shown that simple protein electrophoretic surveys of Mb can provide useful data for littorinid systematics and population genetic analyses (De Wolf et al. 1998, Medeiros et al. 1998, Olabarria et al. 1998). In this context, Medeiros et al. (1998) observed that within the genus *Littorina* there was considerable intra- and interspecific Mb differentiation. This allowed, for example, to separate the sibling rough periwinkles *Littorina (Neritrema) saxatilis* and *L. (N.) arcana*. In contrast, the Mb patterns of two *Littoraria* species and three *Nodilittorina* species were almost, if not completely, identical. Interestingly, the Mb profile of *L. striata* was similar to that of *Littoraria* spp. and *Nodilittorina* spp., but differed conspicuously from that of *Littorina* spp. This latter result could be interpreted in two, ways: either *L. striata* is not a *Littorina* or the Mb profile of *L. striata* represents a plesiomorphic condition within *Littorina*.

The present contribution is a follow-up of the work by Medeiros et al. (1998). In particular, we will: (1) test the reliability of Mb as species marker to differentiate between *L. saxatilis* and *L. arcana*, (2) compare the Mb profile of *L. striata* with those of three other basal *Littorina* species, which are supposed to represent its closest relatives (*L. keenae*, *L. scutulata* and *L. plena*) (Reid 1990, Reid 1996, Reid et al. 1996), and (3) evaluate the electrophoretic Mb monomorphism in *Littoraria* and *Nodilittorina* (and related genera) by resolving Mb patterns in two additional species (*Nodilittorina hawaiiensis* and *Cenchritis muricatus*).

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Throughout this article we will follow the taxonomy and nomenclature proposed by Reid (1989, 1996). We will use the abbreviation '*L.*' for the name *Littorina*, whereas the name *Littoraria* will be written in full.

MATERIALS AND METHODS

Electrophoretic profiles of radular Mb (and other structural proteins in the radular muscle) were surveyed in 288 periwinkles representing 10 species (Table 1). After collection specimens were transported alive or in liquid nitrogen to the laboratory, where they were stored at −80°C. Sample preparation was as described by Medeiros et al. (1998) and adapted in order to reduce possible artificial Mb variation caused by oxidative denaturation (e.g. Di Iorio 1981, Righetti 1983). Therefore individual radular tissue homogenates were prepared by thawing frozen snails, crushing their shells and dissecting the radular muscle in cold distilled water. The radular muscle was then blotted on filter paper and homogenized in a 0.1% KCN (w/v) in 20% (v/v) glycerol solution, in a ratio of 20μl solution per mg tissue. KCN converts Mb to cyanometmyoglobin, which is more stable and prevents denaturation to hemichromes (Atassi 1964, Di Iorio 1981). Crude homogenates were subsequently centrifuged for 30 min at 27200 × *g* (15000 r.p.m.) at 4°C. The resulting supernates were stored at −80°C until used for electrophoresis.

Vertical polyacrylamide gel electrophoresis (PAGE) was performed in 80 × 80 × 0.75 mm gels ('Mini Protean II' apparatus of Biorad) with a gel strength of 7% and using a discontinuous buffer system with Tris/HCl pH 9.0 as gel buffer and Tris/Glycine pH 9.0 as tray buffer (Backeljau 1989). Otherwise, PAGE procedures and conditions were as described by Medeiros et al. (1998). The protocols of these authors were also followed to perform horizontal isoelectric focusing (IEF) in pH gradients 3–9 and 4–6.5. PAGE and IEF gels were stained for general proteins (including Mb) with Coomassie Brilliant Blue and specifically for Mb with a benzidine recipe (Medeiros et al. 1998).

TABLE 1.

List of littorinid species and collection sites screened for Mb variation

Species	Locality	*N*
Melarhaphe neritoides (Linnaeus, 1758)	Pico, Azores	10
Cenchritis muricatus (Linnaeus, 1758)	Isla Margarita, Venezuela	15
Nodilittorina hawaiiensis Rosewater & Kadolsky, 1981	Hawaii	15
Littorina (Liralittorina) striata King & Broderip, 1832	São Miguel, Azores	15
	Pico, Azores	15
	Terceira, Azores	15
	Madeira	4
Littorina (Planilittorina) keenae Rosewater, 1978	San Simeon, CA, USA	15
	Leo Carrillo Beach, CA, USA	15
	Morro Bay, CA, USA	15
Littorina (Littorina) plena Gould, 1849	Leo Carrillo Beach, CA, USA	15
	Morro Bay, CA, USA	15
Littorina (Littorina) scutulata Gould, 1849	Morro Bay, CA, USA	4
Littorina (Neritrema) compressa Jeffreys, 1865	Port Bhéal an Duin, Ireland	15
	Trébeurden, France	15
Littorina (Neritrema) arcana Hannaford Ellis, 1978	Ravenscar, UK	15
	Robin Hoods Bay, UK	15
Littorina (Neritrema) saxatilis (Olivi, 1792)	Venice, Italy (type loc.)	15
	Robin Hoods Bay, UK	15
	Ravenscar, UK	15
	São Miguel, Azores	15

RESULTS

All specimens of *L. saxatilis*, *L. arcana* and *L. compressa* had the same monomorphic Mb profile, both with PAGE (Fig. 1) and IEF (Fig. 2). These profiles corresponded with the *L. saxatilis* profile reported by Medeiros et al. (1998), while the alternative Mb profile, said to be typical of *L. arcana* (Medeiros et al. 1998: figs 2, 4), was not observed here.

Both PAGE (not shown) and IEF revealed that *L. scutulata* and *L. plena* have identical Mb profiles, which resemble that of *L. saxatilis*, except for the fact that with IEF the whole *L. saxatilis* Mb profile was slightly shifted toward a higher pH (Fig. 3). *L. keenae*, on the contrary, had a very different Mb profile which was shared with *L. striata*. Yet, with IEF *L. striata* revealed an additional Mb fraction at a pI of about 4.6, which was not observed in the other littorinids studied here (Fig 3). On the other hand, *L. striata* and *L. keenae* showed a more or less strong Mb band at a pI around 6.3. This band was lacking in the subgenera *Littorina* and *Neritrema*, but was present in *N. hawaiiensis*, *C. muricatus* and *M. neritoides*. For the remainder the IEF profiles of *N. hawaiiensis* and *C. muricatus* were similar to those of *L. keenae* and *L. striata*, although they did not show the Mb fraction at pI 4.6 of *L. striata* (Fig. 4). Finally, similar to the results of Medeiros et al. (1998), the Mb profile of *M. neritoides* was indistinguishable from that of *L. striata* with PAGE (not shown), but appeared to be distinct with IEF (Fig. 3).

Surprisingly, in contrast to the apparent Mb monomorphism of *C. muricatus* revealed by IEF, PAGE of the same individuals yielded a tentative Mendelian polymorphism reminiscent of a monomeric protein coded by a single locus with two alleles (Fig. 5).

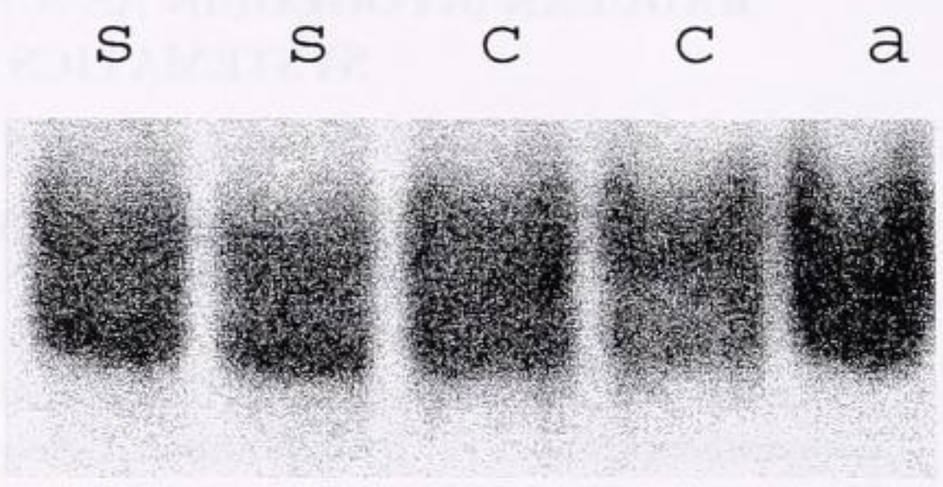

Figure 1. Benzidine staining of PAGE profiles of radular Mb in *L. arcana* (a), *L. compressa* (c) and *L. saxatilis* (s).

DISCUSSION

The present analyses show that the alleged species specific Mb differentiation between *L. saxatilis* and *L. arcana* (Medeiros et al. 1998) is not foolproof, since the *L. saxatilis* profile also occurs in *L. arcana*. Hence, as Medeiros et al. (1998) screened only one population of *L. arcana* (from Great Castle Head near Dale Fort, UK) and did not apply the KCN protocol to reduce artificial Mb variation, it seems worthwhile to screen a new batch of animals from this population in order to confirm the existence of two electrophoretic Mb types in *L. arcana*.

The fact that *L. scutulata* and *L. plena* have identical Mb patterns is not surprising, given the close relationship between both species (Mastro et al. 1982, Murray 1982, Reid 1996, Rugh 1997). It is however, interesting that the Mb profile of these two species differs from that of *L. saxatilis*, *L. arcana*, *L. compressa*, and *L. littorea* (this latter by inference from Medeiros et al. 1998), while

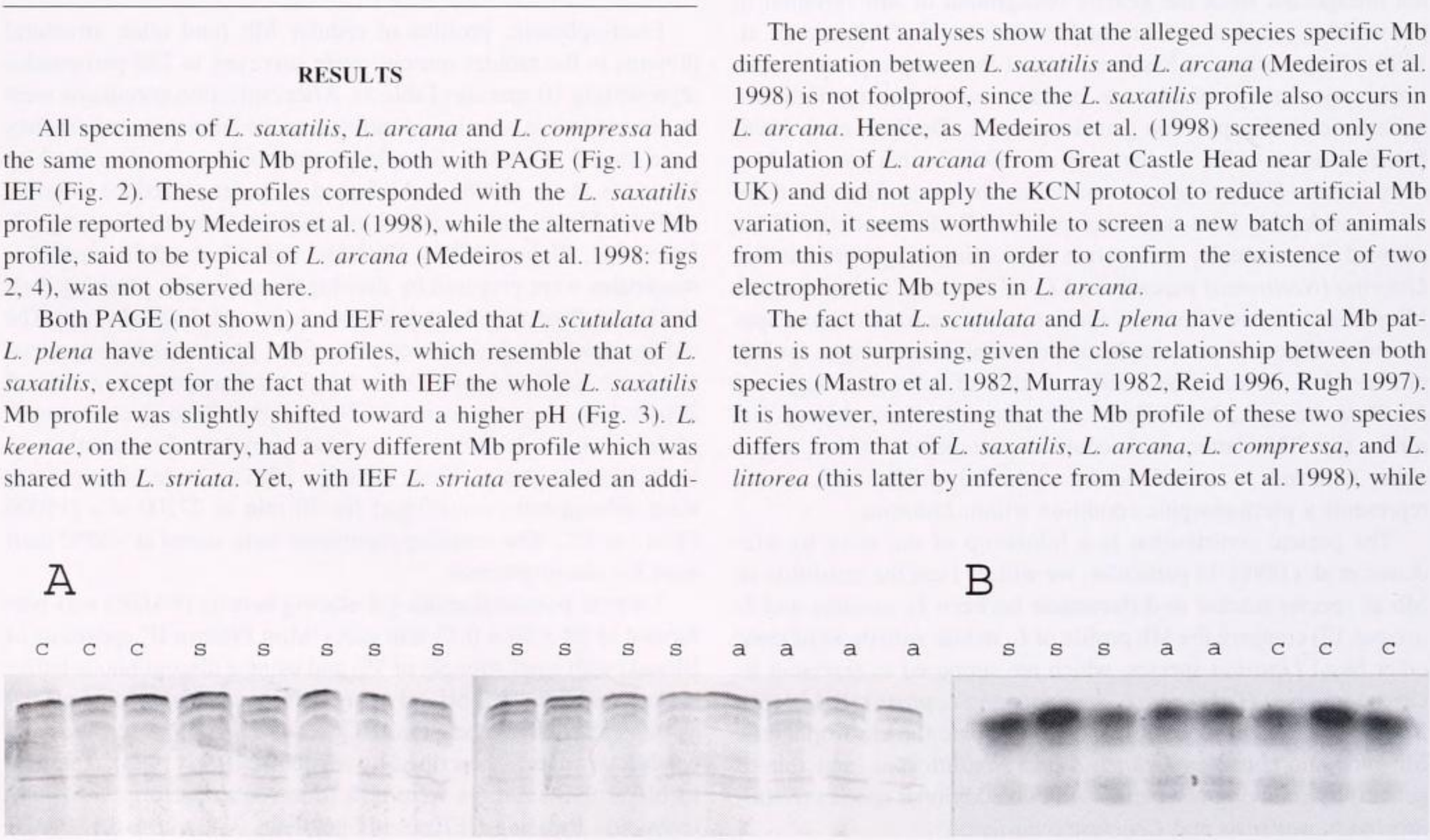

Figure 2. IEF profiles (pH 4–6.5) of radular Mb in *L. arcana* (a), *L. compressa* (c) and *L. saxatilis* (s), stained with Coomassie Brilliant Blue (A) and benzidine (B).

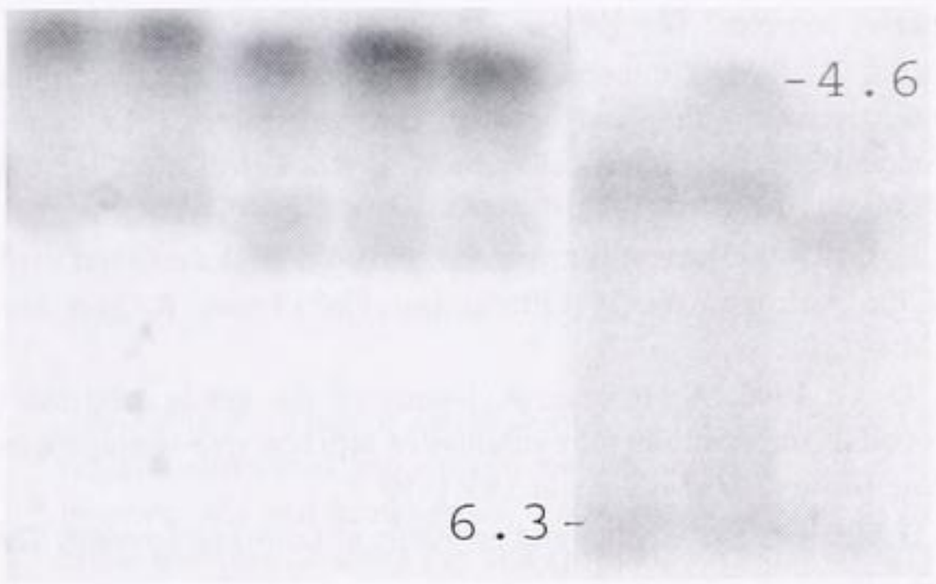

Figure 3. Benzidine staining of IEF profiles (pH 4-6.5) of radular Mb in *L. arcana* (a), *L. compressa* (c), *L. keenae* (k), *M. neritoides* (n), *L. plena* (p), *L. striata* (r), *L. saxatilis* (s), and *L. scutulata* (u). The arrow indicates the special band at pH 4.6 in *L. striata*; the triangle indicates the band at pH 6.3.

the Mb profiles of *L. keenae* and *L. striata* differ even more conspicuously from the Mb profile of these four species. Instead the Mb profiles of *L. keenae* and *L. striata* are similar to those of *Nodilittorina* spp., *Littoraria* spp. and *Cenchritis* sp. reported here and by Medeiros et al. (1998). These data are consistent with current taxonomic practice to place *L. striata* and *L. keenae* in the separate monotypic subgenera *Liralittorina* and *Plan ilittorina* (Reid 1996), such that the Mb pattern of *L. keenae* could have been derived from that of *L. striata* by the loss of the supposedly autapomorphic Mb band at pI 4.6 in *L. striata*. The Mb profile of *L. scutulata* and *L. plena* would then represent a synapomorphy distinguishing both species from the other *Littorina* and *Neritrema* species, while Mb profiles of *L. littorea*, *L. saxatilis*, *L. compressa* and *L. arcana* would unite the subgenera *Littorina* and *Neritrema*. Finally, the Mb patterns reported by Medeiros et al. (1998) for *L. arcana* (if correct), *L. fabalis* and *L. obtusata* may involve still further derived states. Given that in the consensus phylogeny of the genus *Littorina* (Reid 1996: Fig. 119) *L. scutulata* and *L. plena* form an independent clade (making the subgenus *Littorina* paraphyletic), our Mb data support the suggestion that these latter two species may constitute a separate subgeneric group.

Although this scenario fits the currently accepted phylogeny of the genus *Littorina*, the Mb patterns are also compatible with the alternative idea that *L. striata* may be not a *Littorina*. Yet, in that case *L. keenae*, whose assignment to *Littorina* has never been challenged, would become the most basal branch of the genus, suggesting that a *Nodilittorina/Littoraria*-like Mb profile (like in *L. striata* and *L. keenae*) is not a priori inconsistent with the genus *Littorina*. However, since electrophoretic mobilities are not reliable to infer homology and/or identity, the present Mb data are essentially phenetic. Hence apparent similarities may not necessarily indicate common descent, particularly not in a molecule like Mb which may be subject to functional constraints that may cause homoplasy. Nevertheless, the Mb data do suggest that a sequence analysis at the amino acid and nucleotide level could help to understand littorinid phylogeny and functional ecology. Such studies have been done for abalones (Suzuki et al. 1997) and anaspids (Rinaldi & Ophir 1998)

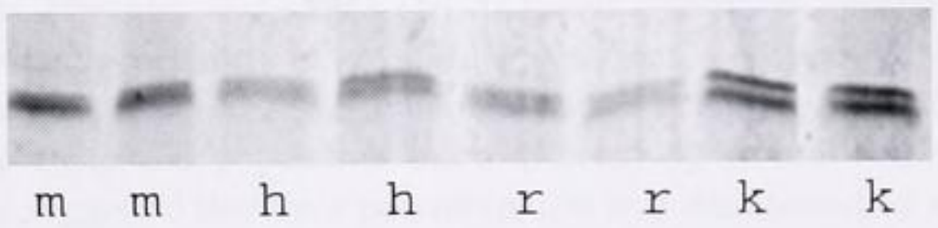

Figure 4. Coomassie Brilliant Blue staining of IEF profiles (pH 4-6.5) of *N. hawaiiensis* (h), *L. keenae* (k), *C. muricatus* (m), and *L. striata* (r).

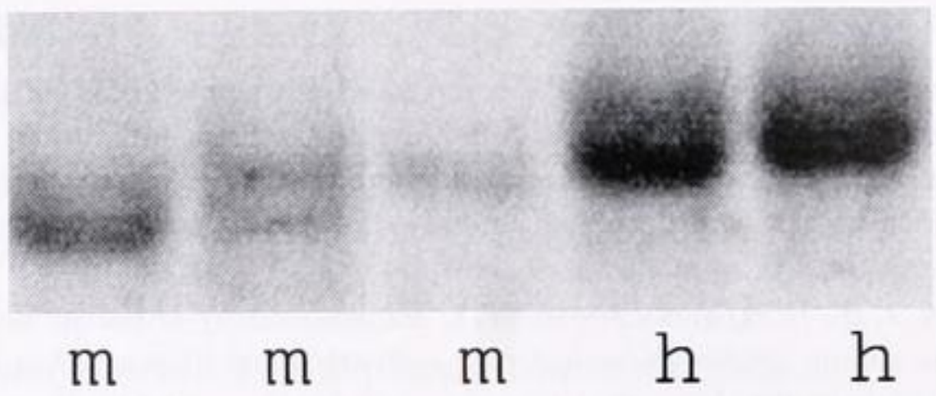

Figure 5. Benzidine staining of PAGE profiles of radular Mb in *C. muricatus* (m) and *N. hawaiiensis* (h). Note the suggestive Mendelian-like variation in *C. muricatus*.

Finally, the IEF Mb data on *N. hawaiiensis* and *C. muricatus*, add to the Mb monomorphism in the genera *Littoraria* and *Nodilittorina* (Medeiros et al. 1998) and extend this observation to the genus *Cenchritis*. However, they contradict the alleged species specific Mb variation in *Littoraria* and *Nodilittorina* as reported by Jones (1972), who used PAGE. Probably this discrepancy is due to technical issues and/or hidden Mb heterogeneity. Indeed, when we applied PAGE in *C. muricatus*, we also detected variation that was not uncovered by IEF. Hence, combined with the observations of Medeiros et al. (1998) on the hidden PAGE differentiation in *M. neritoides* that was resolved by IEF, it is obvious that PAGE and IEF are complementary in the analysis of littorinid Mb variation. However, whether the *C. muricatus* patterns really involve a Mendelian polymorphism, needs further scrutiny in view of the confusing evidence on the genetic background of littorinid Mb (Olabarria et al. 1998).

In conclusion, the present data confirm Medeiros et al.'s (1998) claim that littorinid Mb are useful systematic markers that can provide the same kind of information as do the haemoglobins in freshwater snails (Bailey et al. 1986) and fishes (e.g. Basaglia & Callegarini 1987, Macaranas et al. 1996, Rizzotti & Gioppato 1999), or the haemocyanins in terrestrial gastropods (e.g. Symondsen & Walton 1994) and crustaceans (e.g. Mangum 1996, Mangum & McKenney 1996). Nevertheless, littorinid Mb are less suited to differentiate closely related species, but on the other hand seem quite informative for higher level systematics. Unfortunately, the present knowledge on littorinid Mb is still far too scanty to fully exploit them for ecophysiological, population genetic, taxonomic and phylogenetic investigations.

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