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Radular myoglobin and protein variation within and among some littorinid species (Mollusca: Gastropoda)

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Abstract

The radular muscles of several littorinid species, including *Littorina littorea*, *L. saxatilis*, *L. obtusata*, *L. striata* and *Melarhaphe neritoides*, contain myoglobin (Mb). Here we report on the presence of radular Mb in eight other littorinids: *L. compressa*, *L. arcana*, *L. fabalis*, *Nodilittorina punctata*, *N. trochoides*, *N. radiata*, *Littoraria undulata* and *Littoraria cingulifera*. Using native polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) we compared the Mb and soluble protein (SP) profiles of these species. This suggested that: (1) *L. saxatilis* and *L. arcana* may have specific Mb/SP profiles, (2) *Littoraria* spp., *Nodilittorina* spp. and *L. striata* share similar Mb patterns, (3) Mb is remarkably diverse in the genus *Littorina*, (4) *L. littorea* shows intraspecific Mb/SP variation, (5) *L. saxatilis* does not show geographic Mb/SP differences, and (6) IEF uncovers substantial hidden Mb/SP heterogeneity not shown by PAGE (particularly for *Melarhaphe neritoides*). Hence, littorinid Mb/SP may be a useful taxonomic marker whose ecophysiological significance deserves further study, even if its genetic basis remains unclear.

Introduction

Although several prosobranch gastropods are known to contain myoglobin (Mb) in their radular muscles (e.g. Read, 1966; Bonaventura & Bonaventura, 1983), data on the presence of radular Mb still need to be collected for the majority of species (Bonaventura & Bonaventura, 1983). Yet, within the Littorinidae (periwinkles), the occurrence of radular Mb has been demonstrated in at least 17 species, including *Littorina littorea*, *L. saxatilis* (formerly referred to as *L. rudis*), *L. obtusata*, *L. striata*, *Melarhaphe neritoides* and several species of *Nodilittorina* and *Littoraria* (Read, 1966, 1968; Bannister et al., 1968; Wium-Andersen, 1970; Jones, 1972; Alyakrinskaya, 1986, 1989, 1994). Despite the relative wealth of data on littorinid Mb only two authors, viz. Wium-Andersen (1970) and Jones (1972), have attempted to use Mb variation in littorinid population genetics and systematics.

Wium-Andersen (1970) applied native polyacrylamide gel electrophoresis (PAGE) to compare radular Mb profiles of *L. striata*, *L. littorea* and *L. saxatilis*. The results of this work suggested that *L. striata* and *L. littorea* have monomorphic Mb profiles with *L. striata* being fixed for the two slow migrating bands and *L. littorea* for the two fast migrating bands. Furthermore, it appeared that *L. saxatilis* shows Mendelian variation of the two Mb bands observed in *L. littorea*, such that (1) genotype frequencies in Danish populations show a nice fit to those expected under Hardy-Weinberg equilibrium conditions and (2) Mb gene frequencies follow a cline along the Danish west coast. Finally, Wium-Andersen (1970) reported that radular proteins other than Mb, are fixed (but different) in *L. striata*

and *L. littorea*, whereas in *L. saxatilis* they are highly variable, suggesting a strong (species-specific?) divergence of *L. groenlandica*, but no clear separation of *L. tenebrosa* [currently the latter two taxa are considered as varieties or ecotypes of *L. saxatilis* (Reid, 1996)].

Jones (1972) also used native PAGE of radular Mb in a systematic study of 11 Panamanian '*Littorina*' species (currently all these species are assigned to *Nodilittorina* and *Littoraria*; David Reid in litt., 1996). The aim of this work was to identify so-called Pacific-Caribbean 'geminate' (twin or sister) species on both sides of the Panamanian Isthmus. However, although Jones (1972) observed species-specific differences in both the mobility and the number of Mb bands, she could not define 'geminate' species pairs. Moreover, the phylogenetic inferences based on Jones' (1972) data were inconsistent (Reid, 1986).

Nevertheless, the appealing Mb results of Wium-Andersen (1970), combined with the fact that haemoglobin/myoglobin polymorphisms have been used in the systematics and population genetics of bivalves (e.g. O'Gower & Nicol, 1968), basommatophoran gastropods (e.g. Bailey et al., 1986; Mulvey et al., 1988; Woodruff & Mulvey, 1997) and other animal groups (e.g. Basaglia & Callegarini, 1987; Lavrenchenko et al., 1992; Alexandrino et al., 1996; Das & Handique, 1996; Macaranas et al., 1996), prompted us to initiate an electrophoretic survey of littorinid Mbs. Therefore we aimed at: (1) extending Wium-Andersen's (1970) observations, (2) screening a number of species for which the occurrence of Mb was not yet documented, (3) assessing the degree of intraspecific variation of littorinid Mbs, (4) testing the utility of Mb (and other radular proteins) as markers to distinguish closely related species, (5) evaluating the utility of Mb for supraspecific littorinid systematics, and (6) comparing the power of different electrophoretic methods to detect Mb variation.

Throughout this paper we will follow the taxonomy and nomenclature proposed by Reid (1986, 1992, 1996). Furthermore, we will maintain the abbreviation '*L.*' for *Littorina*, while the name *Littoraria* will be written in full.

Materials and methods

Radular muscle Mb and soluble proteins (SP) were investigated in 482 specimens from 19 populations involving 11 littorinid species. Two additional species were represented by pooled samples of about 20 individuals each. The following material was included: *Melarhaphe neritoides* (Linnaeus, 1758): São Miguel (Azores) (No. = 10); *Littoraria cingulifera* (Dunker, 1845): Gorée (Senegal) (No. = 10); *Littoraria undulata* (Gray, 1839), Laing Island (Papua New Guinea) (No. = 10); *Nodilittorina (N.) trochoides* (Gray, 1839) [formerly *N. pyramidalis* (Quoy & Gaimard, 1833)], Cape d'Aguilar (Hong Kong) (No. = 20 pooled); *N. (N.) radiata* (Eydoux & Souleyet, 1852) [formerly *N. exigua* (Dunker, 1860)], Cape d'Aguilar (Hong Kong) (No. = 20 pooled); *N. (Echinolittorina) punctata* (Gmelin, 1791), Brucoli (Sicily) (No. = 10); São Vicente (Cape Verde Islands) (No. = 10), *Littorina (Liralittorina) striata* King & Broderip, 1832, São Miguel (Azores) (No. = 10); *L. (Neritrema) obtusata* (Linnaeus, 1758), Roscoff (France) (No. = 10); *L. (Neritrema) fabalis* (Turton, 1825) [formerly *L. mariae* Sacchi & Rastelli, 1966], Zeebrugge (Belgium) (No. = 10); *L. (Neritrema) compressa* Jeffreys, 1865 [formerly *L. nigrolineata* Gray, 1839], Roscoff (France) (No. = 10); *L. (Neritrema) arcana* Hannaford Ellis, 1978, Dale Fort 'Great Castle Head' (U.K.) (No. = 40); *L. (Neritrema) saxatilis* (Olivi, 1792), Trondheim (Norway) (No. = 40), Ria de Arosa (Spain) (No. = 40); *L. (Littorina) littorea* (Linnaeus, 1758), Brouwersdam (The Netherlands) (No. = 40), Oostende (Belgium) (No. = 38), Wimereux 'Dique de la Crèche' (France) (No. = 40), Ile de Ré (France) (No. = 40), Ria Ferrol (Spain) (No. = 40), Ria de Arosa (Spain) (No. = 40), Milford Haven (U.K.) (No. = 34).

After field collection, all material was transported alive or frozen in liquid nitrogen to the laboratory where it was stored at –80 °C until prepared for electrophoresis. Prior to sample preparation all specimens were sexed. In *L. littorea* and *L. saxatilis* five shell measurements were taken with vernier callipers: height, width, height of the aperture, width of the aperture and height of the shell top. In both species the colour pattern and total weight were determined. Individual tissue homogenates were prepared by thawing frozen snails, crushing their shells and dissecting the radular muscles in cold distilled water. The muscles were blotted on filter paper and, depending on the tissue volume, transferred into 40, 25 or 15 μl of a 20% (w/v) aqueous sucrose solution, in which the muscles were homogenised. Crude homogenates were centrifuged for 45 min at 27000 × ***g*** and 4 °C. The resulting supernates were stored at –80 °C until used for electrophoresis.

Native vertical PAGE was performed in 80 × 80 × 0.5 mm gels ('Mighty Small SE 250' apparatus

Table 1. Programmed conditions for IEF separations of Mb/SP in polyacrylamide gels (pH gradient: 3–9) using PhastSystem. The conditions for 4–6.5 pH gradients were the same except that SEP 1.1 and SEP 1.2 were set at 2.0 mA, while SEP 1.3 was set at 5.0 mA

SAMPLE APPLICATOR DOWN AT				1.2	0 Vh
SAMPLE APPLICATOR UP AT				1.3	0 Vh
EXTRA ALARM TO SOUND AT				1.1	73 Vh
SEP 1.1	2000 V	2.5 mA	3.5 W	5 °C	75 Vh
SEP 1.2	200 V	2.5 mA	3.5 W	5 °C	15 Vh
SEP 1.3	2000 V	2.5 mA	3.5 W	5 °C	410 Vh

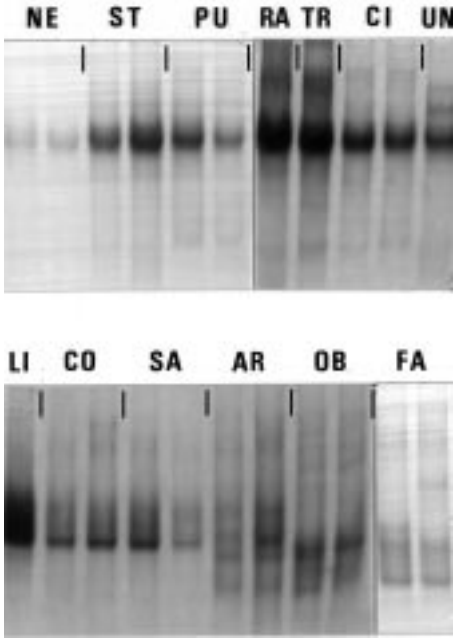

Figure 1. Native vertical PAGE profiles (top = cathodal gel side) of Mb in 13 littorinid species. Abbreviations: AR = *L. arcana*; CI = *Littoraria cingulifera*; CO = *L. compressa*; FA = *L. fabalis*; LI = *L. littorea*; NE = *M. neritoides*; OB = *L. obtusata*; PU = *N. punctata*; RA = *N. radiata*; SA = *L. saxatilis*; ST = *L. striata*; TR = *N. trochoides*; UN = *Littoraria undulata.*

of Hoefer Scientific Instruments), with a gel concentration of 7% and using a discontinuous buffer system consisting of Tris-HCl (pH 9.0) in the gel and Tris-Glycine (pH 9.0) in the tray (Backeljau, 1989). Electrophoreses were started at 25 V for 15 min and continued at 150 V until the migrating front reached the anodal (lower) gel side. During the experiments, temperatures were kept below 15 °C. Depending on the concentration of the homogenates (roughly estimated by the intensity of the red colour), 1–7 μl of sample was applied per run.

Horizontal IEF was performed in precast mini polyacrylamide gels of 50 × 43 × 0.35 mm containing a 3–9 or a 4–6.5 pH gradient. Gels were run on LKB-Pharmacia's PhastSystem (Olsson et al., 1988; Backeljau et al., 1994), according to the programmed conditions outlined in Table 1 and with the sample applicator positioned in the cathodal region of the gels. IEF runs took 30–45 min.

After IEF, soluble proteins were stained with Coomassie Brilliant Blue R-250. Therefore, gels were first placed for 15 min in an aqueous solution containing 5% (w/v) 5-sulfosalicylic acid and 10% (w/v) trichloroacetic acid. Subsequently, gels were rinsed for 2 × 10 min in an aqueous stock solution containing 35% (v/v) ethanol and 10% (v/v) glacial acetic acid. Next, gels were stained for 5 min in stock solution in which 0.2% (w/v) Coomassie Brilliant Blue R-250 was dissolved. Finally, gels were destained for 2 × 10 min in pure stock solution. Coomassie staining of PAGE gels was done in the same way, but with each step taking more time.

Mb bands were identified by a specific and sensitive benzidine staining (Broyles et al., 1979). To this end gels were placed for 10 min in a benzidine solution (0.4 g benzidine dissolved in 200 ml of a 1:1:1 mixture of acetic acid, ethanol and water), after which they were transferred into a 2% (v/v) aqueous hydrogen peroxide solution. As benzidine is a highly potent carcinogen (Broyles et al., 1979; Riggs, 1981), this staining procedure was only used for the initial detection of Mb in the profiles and for the evaluation of new patterns.

Given the preliminary nature of the present analyses, we neither quantified protein concentrations, nor tried to infer exact molecular weights or isoelectric points. Yet, such data are not a prerequisite for systematic comparisons (e.g. Macaranas et al., 1996;

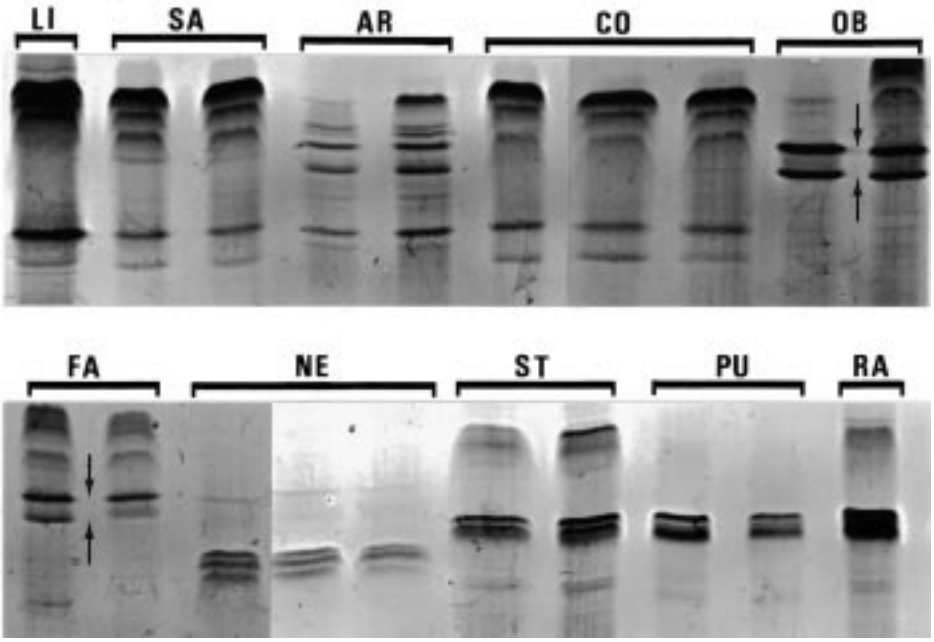

Figure 2. IEF patterns (top = anodal gel side) in a 4–6.5 pH gradient of Mb/SP in nine littorinid species. Abbreviations as in Figure 1. The bands marked by an arrow serve as references.

Mangum, 1996; Mangum & Greaves, 1996; Mangum & McKenney, 1996).

Results

All species studied had conspicuously red radular muscles suggesting the presence of Mb. This red colour remained visible during, but disappeared soon after, electrophoresis. Yet, in all cases the presence of Mb was further supported by a strong benzidine staining reaction (Figures 1, 3A).

PAGE was unable consistently to resolve protein bands other than Mb. Yet, it did reveal four different, more or less well-resolved Mb patterns (Figure 1): (1) a single, monomorphic, slow migrating (cathodal) band shared by *M. neritoides*, *Littoraria* spp., *Nodilittorina* spp. and *L. striata*, (2) a nearly monomorphic two-banded, faster migrating pattern shared by *L. littorea*, *L. compressa* and *L. saxatilis*, (3) a complex pattern with a partially more anodal position in *L. obtusata* and *L. fabalis*, and (4) a unique, distinct three- to four-banded pattern in *L. arcana*.

Although in two-banded PAGE patterns, the more cathodal band was sometimes poorly resolved (Figure 1), we found little or no Mb variation within or among the seven *L. littorea* populations or the two *L. saxatilis* populations. In *L. arcana* we detected one individual with four bands instead of the usual three, while another specimen showed a Mb profile typical of the group *L. saxatilis/L. compressa/L. littorea*. It is possible that this individual was misidentified.

In contrast to PAGE, IEF yielded very well-resolved, but complex Mb/SP patterns, and this with both the 3–9 and 4–6.5 pH gradients. In all species, the Mbs had rather low isoelectric points (estimated pI < 5.0). The IEF profiles of Mb confirmed the four species groups defined by PAGE, except for *M. neritoides*, which revealed a unique fifth Mb pattern that differed from all other species, including those of the *Littoraria/Nodilittorina/L. striata* group (Figure 2). In this latter group, IEF demonstrated SP differences between the two *Littoraria* species, as well as between *L. striata*, *N. punctata* and *N. radiata*. However, this latter species could not be distinguished from *N. trochoides*. Similarly, *L. obtusata* and *L. fabalis* could not be separated by their Mb patterns (see Olabarria et al., this volume), but both species were strongly differentiated from any other littorinid included in this work (Figure 2).

IEF also uncovered hidden heterogeneity with respect to intraspecific Mb/SP variation in *L. littorea*. In this species the benzidine staining of the IEF profiles consistently revealed two heavy 'Mb' bands (Figure 3A). Yet, the Coomassie staining showed that both

Table 2. Intraspecific Mb/SP variation in *L. littorea*, showing per population the numbers of individuals with a given overall Mb/SP profile (A–F) as resolved with IEF in a 4–6.5 pH gradient. *N* = total number of specimens investigated

Population (*N*)	A	B	C	D	E	F
Brouwersdam (40)	40	–	–	–	–	–
Oostende (38)	38	–	–	–	–	–
Wimereux (40)	3	–	–	1	36	–
Ile de Ré (40)	36	3	1	–	–	–
Ria Ferrol (40)	40	–	–	–	–	–
Ria de Arosa (40)	39	–	–	–	–	1
Milford Haven (34)	34	–	–	–	–	–

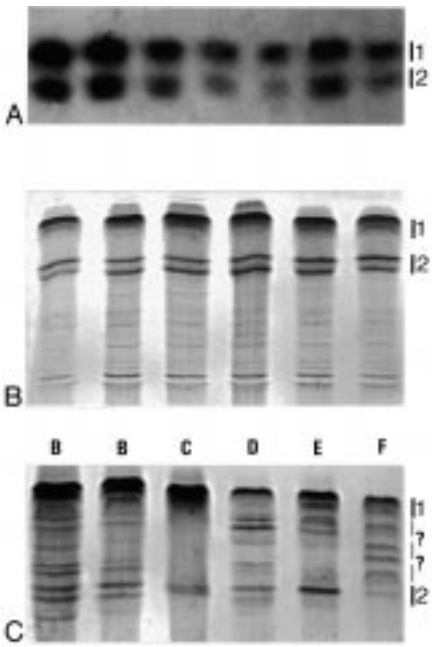

Figure 3. IEF patterns (top = anodal gel side) of Mb/SP variation in *L. littorea*. A. Mb profiles in a 3–9 pH gradient stained by benzidine; B. Most common IEF profile of Mb/SP (pattern A) (3–9 pH gradient) in the Oostende population; C. Variant IEF profiles of Mb/SP resolved in a 4–6.5 gradient. The corresponding zones in the three gels are numbered (1–2), but the bands between the zones cannot be assigned unambiguously

bands consist of several protein fractions with similar pI values. Although some of these fractions must involve Mb, it is likely that others represent SP. Anyway, six different IEF patterns could be distinguished (A–F) with the Mb/SP bands (Figures 3B–C). Pattern A was clearly the common one since four populations (Brouwersdam, Oostende, Milford Haven and Ria Ferrol) were fixed for it (Figure 3B), while in two other populations (Ile de Ré and Ria de Arosa) it was present in more than 90% of the snails (Table 2). In the Wimereux population, however, pattern A was found in only 7.5% of the animals, while 90% of the specimens showed pattern E (one individual had profile D) (Table 2). None of these IEF patterns showed an appreciable Mb/SP variation with PAGE, except for the F profile, which was also quite distinct (one individual) with PAGE. In any case, the observed Mb/SP variation in *L. littorea* could not be correlated with sex, size or colour characteristics of the shell.

In contrast, *L. saxatilis* did not reveal hidden Mb/SP variation with IEF, so that the lack of differentiation between the Norwegian and Spanish populations, as suggested by PAGE, was confirmed by IEF (Figure 4C). Yet, the Mb differentiation between *L. saxatilis/L. compressa/L. littorea* and *L. arcana* was well-resolved and consistent with IEF (Figures 4A–B). The SP profiles reinforced this differentiation.

Finally, it appeared that long term storage at –80 °C did not affect Mb/SP patterns as is shown by the comparison of the profiles of *L. littorea* (1992–1995). Because Wittenberg & Wittenberg (1981) suggested that Mb in 0.05 M Tris-HCl buffer at pH 8.0 remains stable even after repeated freezing and thawing, we compared the stability of *L. littorea* Mb in this buffer (with 1 mM EDTA) and in our 20% (w/v) aqueous sucrose solution. After repeated freezing and thawing, both Mb preparations yielded exactly the same IEF profiles. Similarly, the EDTA experiment suggested that oxidation had little or no effect on our results. Moreover, even if oxidation affected our Mb profiles, we still think that this would hardly influence the systematic implications of our analyses, because all samples were treated in the same way.

Discussion

This study is the first report on the occurrence of radular Mb in *Littoraria undulata*, *Littoraria cingulifera*, *N. punctata*, *N. trochoides*, *N. radiata*, *L. compressa*, *L. arcana* and *L. fabalis*. Yet, the red colour of the radular tissue combined with a strong benzidine reaction is still not a foolproof identification of Mb, so that additional evidence from spectrophotometric absorption spectra would be desirable.

Our PAGE results confirmed only two observations of Wium-Andersen (1970), viz. (1) *L. striata* has a monomorphic Mb electromorph which moves slower than that of *L. littorea* or that of *L. saxatilis*, and (2)

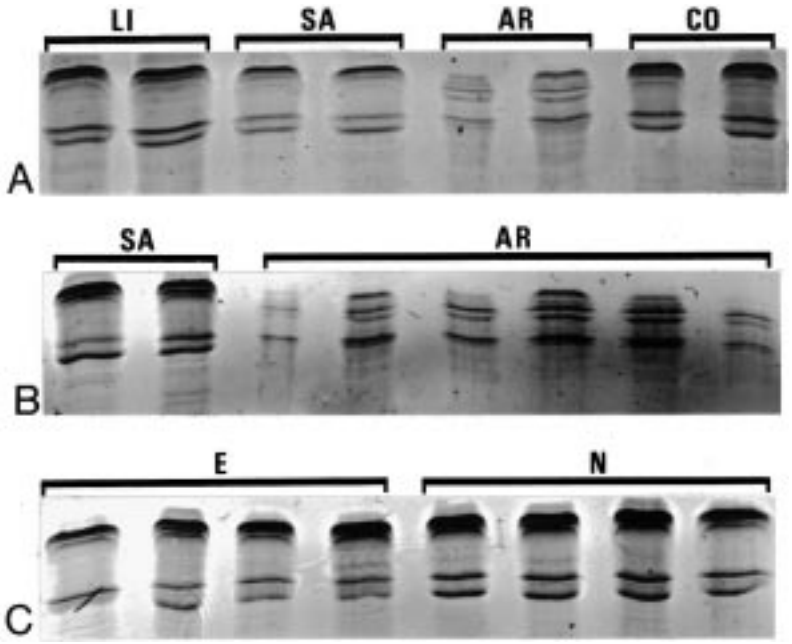

Figure 4. IEF patterns (top = anodal gel side) of Mb/SP in *L. littorea*, *L. saxatilis*, *L. arcana* and *L. compressa* in a 3–9 pH gradient. A–B. Differentiation and consistency of the *L. arcana* pattern; C. Lack of differentiation between *L. saxatilis* from Spain (E) and Norway (N). Abbreviations as in Figure 1.

L. littorea and *L. saxatilis* have a similar two-banded Mb pattern. However, our PAGE results differed from those of Wium-Andersen (1970) by (1) the single Mb band in *L. striata*, (2) the presence of a rare Mb variant in *L. littorea*, and (3) the absence of (Mendelian) variation in the Mb of *L. saxatilis*. Further analyses are required to determine whether these discrepancies are due to technical artefacts (e.g. the single or two banded Mb of *L. striata*), or biases in sample size and geographic origin of the populations studied (e.g. the variation or lack thereof in *L. littorea* and *L. saxatilis*). Anyway, we currently doubt that Wium-Andersen's (1970) Mendelian interpretation of the Mb variation in *L. saxatilis* can be maintained. This interpretation is indeed difficult to reconcile with the fixed two-banded Mb patterns the same author reported for *L. striata* and *L. littorea*, unless (1) both species show fixed heterozygosity at the Mb locus, (2) they have a fundamentally different genetic basis of Mb expression, and/or (3) the quaternary structure of Mb in *L. littorea* and *L. striata* is different from that of rough periwinkles (see Olabarria et al., this volume). In this context it is worth mentioning that the Mb of *L. littorea* is reported to be a dimer, that at low concentrations mainly exists as a dissociated monomer (Read, 1968; Terwilliger & Read, 1969).

Although IEF yielded a higher resolution than PAGE, it was difficult, if not impossible, to interpret the genetic background of the complex banding patterns (see Olabarria et al., this volume). The increased number of Mb bands observed with IEF may be due to several factors. Firstly, electrophoresis or IEF of homogeneous preparations of ferric Mb often yield many fractions with identical amino acid compositions, ligand kinetics and conformations, but different charges (e.g. Rumen, 1959; Edmundson & Hirs, 1962; Atassi, 1964; Parkhurst & LaGow, 1975; Wittenberg & Wittenberg, 1981). Yet, these fractions are not artefacts of the isolation procedure or proteolysis in the muscle homogenates (Wittenberg & Wittenberg, 1981). Multiple Mb bands can also be caused by *in vitro* auto-oxidation (Righetti, 1983), because fully oxidised, partially oxidised and reduced Mb may have different charges. Thirdly, complex Mb band profiles may also result if the dissociation products of the dimeric littorinid Mb have different isoelectric points. Since we diluted our samples it is possible that (part of) the Mb was dissociated. Anyway, under physiological conditions some haemoglobins are known to dissociate readily and reversibly (Righetti, 1983) and even human myoglobin, which is definitely a monomer, may produce multiple fractions with different pI values (e.g. Giraldino

et al., 1995). Hence, in the absence of further data, the interpretation of the *L. littorea* Mb polymorphism remains unclear. However, we suspect that at least part of the observed intraspecific Mb variation may have an ecophysiological significance reflecting feeding regimes, intertidal zonation patterns, pollution, etc. Alyakrinskaya (1986, 1989, 1994), for example, related differential Mb concentrations in prosobranch radular muscles with feeding and activity patterns, while Bonaventura et al. (1975) more generally emphasised the ecophysiological significance of multiple haemoglobins and haemoglobin polymorphisms in vertebrates. Anyway, the *L. littorea* Mb/SP polymorphism strongly contrasts with some allozyme data that suggest a high genetic homogeneity among European *L. littorea* populations (e.g. Johannesson, 1992). Nevertheless, Johannesson (1992) remarked (1) that there was a handful of genotypically 'abnormal' specimens along the Belgian coast, and (2) that the population from Roscoff (France) studied by Berger (1977) may be an 'odd' representative of European *L. littorea*. This led Johannesson (1992) to the very speculative suggestion that two sibling species may be involved. Without expanding on this issue, it is noteworthy that in our study it was the two French *L. littorea* populations that showed the most conspicuous Mb/SP heterogeneity.

Another promising result of our Mb/SP data was the suggestive diagnostic differentiation between *L. arcana* and *L. saxatilis/L. compressa*. This result was unexpected because, hitherto, no diagnostic protein markers were known for *L. arcana* (review by Reid, 1996). Yet, given the limited number of populations studied and the relatively small sample sizes, it is evident that the value of Mb/SP as a diagnostic marker must be verified on the basis of a more extensive survey of *L. arcana*, *L. saxatilis* and *L. compressa*.

In general, the combination of Mb with SP increased the value of radular protein patterns as taxonomic markers. This was not only evident for the differentiation of *L. arcana* and *L. saxatilis*, but also of *L. littorea* and *L. saxatilis*, which had the same Mb profile, but differed consistently by other radular SP. A similar observation applied to the two *Littoraria* species. However, neither *N. trochoides* and *N. radiata* nor *L. saxatilis* and *L. compressa* could be differentiated during this study (for the *L. obtusata/fabalis* complex see Olabarria et al., (1998)).

The last surprising result of our Mb work was the relative intra- and interspecific Mb diversity in *Littorina* spp. compared to the electrophoretic (PAGE and IEF) Mb monomorphism within and among the *Littoraria/Nodilittorina* species. Obviously, the lack of Mb variation in these latter species needs further confirmation with larger sample sizes, particularly since Jones (1972) found a relatively high degree of 'species-specific' Mb differentiation among Panamanian *Nodilittorina* and *Littoraria* species. The fact that our analyses suggest that the Mb of *L. striata* belongs to the *Littoraria/Nodilittorina* group and thus differs from any other *Littorina* Mb so far studied, emphasises the still somewhat problematic generic assignment and phylogenetic position of this species, which has been variously associated with *Melarhaphe*, *Nodilittorina* or (currently) *Littorina* (for recent discussions see Reid, 1989, 1990, 1996; Backeljau & Warmoes, 1992; Reid et al., 1996). Hence, if the placement of *L. striata* in the genus *Littorina* is correct, then the Mb similarity between *L. striata* and *Littoraria/Nodilittorina* may be a plesiomorphic condition. However, the Mb data of *L. striata* should be interpreted with much caution, because (1) the possibility of hidden heterogeneity cannot yet be ruled out, and (2) they may reflect ecological or functional, rather than phylogenetic, correlates. Reid (1996), for example, remarked that the ecology and radula morphology of *L. striata* resembles that of many *Nodilittorina* species. It is tempting to hypothesise that 'ecological or functional correlations' also account for the strong Mb differentiation of *L. obtusata/L. fabalis* (the only obligatory macrophyte-associated littorinids included here; cf. Reid, 1996) and for the Mb characteristics of *L. littorea*. Yet, it must be emphasised that these issues are highly speculative and thus require more detailed investigations.

In conclusion, although the high expectations raised by Wium-Andersen's (1970) paper were not satisfied, and despite the difficulties with the genetic interpretation of littorinid Mb patterns, our results suggest that the electrophoretic analysis of radular Mb/SP may provide a convenient taxonomic marker that can be used in a similar way as haemocyanins (e.g. Symondson & Walton, 1994; Mangum, 1996; Mangum & Greaves, 1996; Mangum & McKenney, 1996). Moreover the ecological and functional significance of littorinid Mb clearly needs to be investigated further as we suspect that such data may yield valuable insights as to how periwinkles interact with their environment.

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