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Periwinkles *(Gastropoda, Littorinidae)* as a model for studying patterns and dynamics of marine biodiversity

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Abstract

This paper presents a summary synthesis of an international EC-funded research program in which periwinkles are used as a model group to study patterns and dynamics in marine biodiversity. In the first part of the paper, a number of techniques and markers to assess biodiversity in periwinkles are illustrated and evaluated, while protocols for DNA-extraction, Single-Strand Conformation Polymorphisms (SSCP) and PCR-amplification of a number of nuclear and mitochondrial DNA markers are provided. The second part of the paper presents a brief overview of the application of these techniques in the analysis of genetic diversity in two periwinkle taxa with contrasting developmental modes, viz. *Littorina striata* and the *L. saxatilis* complex. Finally, these results are discussed in the light of the implementation of the Evolutionarily Significant Unit (ESU) and Management Unit (MU) concepts in (marine) conservation biology. It is concluded that, although these operational concepts are more appropriate than species level systematics for delineating relevant intraspecific diversity, they still may not adequately represent non-molecular evolutionarily relevant, diversity.

Key-words: Gastropoda, Littorinidae, marine biodiversity, population genetics, taxonomy, molecular systematics.

Résumé

Cet article présente une brève synthèse d'un projet international financié par la CE, dans lequel les littorines sont choisies comme groupe modèle pour l'étude de la structure et la dynamique de la biodiversité marine. Dans la première partie de l'article, on présente une illustration et une évaluation d'un nombre de techniques et de marqueurs moléculaires utilisés dans l'étude de la biodiversité des littorines. De plus on propose des protocoles pour l'extraction d'ADN, pour d'analyses de "Single-Strand Conformation Polymorphisms (SSCP)" et pour l'amplification par PCR d'un nombre de marqueurs d'ADN nucléaires et mitochondriaux. La deuxième partie de l'article donne un bref aperçu de l'application de ces techniques dans l'analyse de la diversité génétique dans deux taxons de littorines avec des modes de développement alternatifs: *Littorina striata* et le complexe de *L. saxatilis*. Finalement, les résultats de ces analyses sont discutés dans le cadre de l'application des conceptes comme les "Evolutionarily Significant Units (ESU)" et les "Management Units (MU)" dans la biologie de conservation (marine). Bien que ces "ESUs" et "MUs" soient meilleurs que la systématique au niveau d'espèces, pour délimiter la diversité intraspecifique, ils ne représentent pas suffisamment la diversité non-moléculaire pertinente du point de vue d'evolution.

Mots-clés: Gastropoda, Littorinidae, biodiversité marine, génétique de populations, taxonomie, systématique moléculaire.

Introduction

Biodiversity can be defined as "The variability among living organisms from all sources, including *inter alia* terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems" (CONVENTION OF RIO, 1992), or as MEFFE & CAROLL (1994) put it "The variety of living organisms considered at all levels, from genetics through species, to higher taxonomic levels, and including the variety of habitats and ecosystems". Many more definitions of biodiversity exist, but all entail the same basic tenet, viz. that biodiversity is the property of living systems to be distinct and variable (SOLBRIG, 1994). It is precisely this property that allows living systems to evolve and adapt in an ever changing environment, and biodiversity therefore represents no less than future evolutionary potential. In addition, biodiversity offers a wide variety of economic, social and cultural benefits, the value of which is increasingly recognized and documented in the literature (e.g. OLDFIELD, 1984; BARBIER & AYLWARD, 1996; LOOMIS & WHITE, 1996; SAGOFF, 1996; BENGTSSON *et al.*, 1997; COSTANZA *et al.*, 1997; EDWARDS & ABIVARDI, 1998; TEN KATE & LAIRD, 1999).

Hence, the current worldwide concerns with respect to global change and the concomitant loss of biodiversity are more than justified, since humans are responsible for possibly the fastest and most drastic wave of extinction in the history of life (WILSON, 1988). Hitherto, this problem has been taken seriously in terrestrial ecosystems, where anthropogenic influences such as habitat fragmentation, deforestation, species translocations, urbanisation, pollution, agricultural practices and overexploitation of natural habitats, are having devastating effects on the biosphere. Biodiversity in marine ecosystems, on the contrary, is usually believed to be much less vulnerable to global extinctions caused by such anthropogenic

factors. Yet, recent compelling evidence increasingly indicates (1) that marine species may be at a far greater risk of extinction than is generally assumed (ROBERTS & HAWKINS, 1998) and (2) that currently there is an enormous loss of local marine biodiversity due to local population declines and extinctions (CARLTON, 1993). However, losses of marine biodiversity remain largely unmeasured because of the relative neglect of marine taxonomy and population genetics (MCKINNEY, 1998). Moreover, marine conservation has much less of a theoretical basis than terrestrial conservation (ALLISON *et al.*, 1998). It is therefore tempting to implement experiences from the latter in the marine realm. However, marine ecosystems differ fundamentally from terrestrial systems, both in their spatio-temporal scales and in the variability of processes (ALLISON *et al.*, 1998). It is therefore no surprise that marine conservation biology lags far behind terrestrial conservation biology (NORSE, 1994). Furthermore, just as terrestrial conservation requires a sound knowledge of the patterns and processes that govern the dynamics (i.e. origin, maintenance and loss) of biological variation, so the development of marine conservation strategies will also need such information.

Against this background, the European Community launched within the 'Marine Science and Technology' (MAST-III) work programme an action plan to support fundamental research aiming at a better understanding of patterns and dynamics in marine biodiversity. One of the supported projects was AMBIOS ('Integrating Environmental and Population Variation: A Model for Biodiversity Studies'), which aimed (1) to evaluate a number of techniques to assess diversity at various taxonomic levels and (2) to provide a comprehensive, integrated picture of how diversity may be generated and patterned in organisms with contrasting life histories and living in a spatio-temporally variable marine environment.

The rationale behind the AMBIOS project was that understanding marine biodiversity requires the consideration of at least five issues: (1) how do we measure diversity, (2) which part of biological variation is genetic and which is ecophenotypic, (3) how is diversity structured in space and time, (4) how are these spatio-temporal patterns generated, maintained and lost, and (5) which measures or operational concepts can be implemented to conserve and manage marine biodiversity. Obviously, points (2), (3) and (4) involve the question as to how reproductive barriers are formed and hence how independent genetic entities (often 'species') arise under field conditions. This in turn refers to the basic problem of delimiting 'species' and the concomitant problem of what kind of evolutionarily relevant units of diversity have to be considered for conservation and management. These taxonomic problems indeed formed an important component of the AMBIOS project.

The most efficient way to achieve the AMBIOS goals was by working on a group of widespread, common organisms, which (1) show considerable intra- and interspecific morphological, behavioural, life-history and population genetic diversity, (2) which are clearly in the process of divergence, and (3) which live in the coastal zone as an example of a spatio-temporally, highly variable, marine environment. Periwinkles (Mollusca, Gastropoda: Littorinidae: genus *Littorina*) are such a group of organisms and were therefore chosen as a model group.

The present contribution is a synthesis of the main research activities, results and conclusions of the AMBIOS project (see also MILL *et al.*, 1998). In particular we will discuss (1) a number of different markers and techniques to assess biodiversity, (2) the patterns and dynamics of diversity in planktonic developing (*L. striata*) vs. direct developing (*L. saxatilis*) periwinkles, (3) phylogenetic patterns in *Littorina*, and (4) the implementation of 'Management Units' (MUs) and 'Evolutionarily Significant Units' (ESUs) sensu MORITZ (1994, 1996) as operational concepts in the conservation and management of marine biodiversity.

Periwinkles as a model group

Littorinid snails are important grazers, and hence structuring agents, in the rocky intertidal. They occur in huge numbers all along the European coasts, where currently nine species are recognized: *Melarhaphe neritoides* (LINNAEUS, 1758), *Nodilittorina (Echinolittorina) punctata* (GMELIN, 1791), *Littorina (Liralittorina) striata* KING & BRODERIP, 1832, *L. (Littorina) littorea* (LINNAEUS, 1758), *L. (Neritrema) obtusata* (LINNAEUS, 1758), *L. (N.) fabalis* (TURTON, 1825), *L. (N.) compressa* JEFFREYS, 1865, *L. (N.) arcana* HANNAFORD ELLIS, 1978 and *L. (N.) saxatilis* (OLIVI, 1792). The first four species have planktonic eggs and larvae, and consequently show substantial gene flow between even very distant populations, so that genetic differentiation is considered to be low (e.g. JOHANNESSON, 1992; BACKELJAU *et al.*, 1995a). Nevertheless, as we will outline further in this paper, this large-scale homogeneity may need to be reconsidered in three of the four species. *L. obtusata*, *L. fabalis*, *L. compressa* and *L. arcana*, in contrast, are egg-layers (REID, 1996), showing considerable population differentiation even over relatively short distances. The taxonomic distinction of the flat periwinkles *L. obtusata* and *L. fabalis* is well-established (SACCHI & RASTELLI, 1966; REID, 1990), even though recent work seems to suggest that *L. fabalis* might in fact comprise two sibling taxa (TATARENKOV & JOHANNESSON, 1998, 1999). Similarly, the taxonomy of *L. compressa* and *L. arcana* is no longer contentious (e.g. RAFFAELLI, 1982; WARD, 1990; REID, 1996). Both of these species belong to the 'rough' periwinkles, as does *L. saxatilis*. However, this last taxon is an extremely variable one containing live-bearing (ovoviviparous) animals. It comprises a number of forms, two of which have, in the past, been accorded species status, i.e. *L. neglecta* BEAN, 1844 (a small 'barnacle dwelling' form with a smooth, glossy and nearly globular shell, usually with an obtuse point and a large last whorl covered with numerous transverse, dark, interrupted bands) (e.g. REID, 1993) and, *L. tenebrosa* (MONTAGU, 1803) (a lagoonal form). Although *L. tenebrosa* can apparently live in the same lagoons as *L. saxatilis* s.s., it differs from the latter both morphologically and behaviourally since it is smaller, has a fragile and smooth shell and lives permanently submerged on macrophytes (MUUS, 1967). Nevertheless, BARNES (1993)

considers that it should be afforded only 'variety' status, i.e. *L. saxatilis* var. *lagunae*.

Three other forms of rough periwinkles are here further referred to as *L. saxatilis* H, *L. saxatilis* M, and *L. saxatilis* B. The H form lives high on the shore, has a thin shell, is patulous and produces large embryos. The M form, in contrast, lives on the mid shore, has a massive, thick shell with a small aperture and produces smaller embryos (HULL *et al.*, 1996). Sometimes intermediates between these two forms are found and such individuals produce two size classes of embryos, corresponding to those of forms H and M. Moreover, a high proportion of embryos from intermediate specimens abort, suggesting that the intermediates are real hybrids and that there may be a barrier to gene flow between *L. saxatilis* H and M (HULL *et al.*, 1996). *L. saxatilis* B is a 'barnacle dwelling' form that lives sympatrically with *L. neglecta*, from which it is distinguished by its sculptured, non-banded shell with a an ovoid aperture. Generally, form B resembles *L. saxatilis* s.s. but matures at a smaller size. Part of the AMBIOS work focused on the biological interpretation of different forms within *L. saxatilis*.

In addition to this morphological diversity, *L. saxatilis* shows a vast geographic distribution and occupies a very wide range of habitats from extremely wave-exposed shores to salt marshes and isolated, brackish lagoons. All other European littorinids have much more restricted habitat niches and/or distributional ranges. As the AMBIOS project mainly dealt with *Littorina* species we refer to REID (1996) for a comprehensive and up to date background on the biology, taxonomy and phylogeny of these animals.

Materials and Methods

The AMBIOS study concentrated on two of the planktonic breeders, *L. striata* and *L. littorea*, on two oviparous species, *L. arcana* and *L. compressa*, and on the ovoviviparous *L. saxatilis* complex. A number of specific studies involved still other species.

AMBIOS fieldwork was carried out at a number of key sites such as Filey Brigg, Robin Hoods Bay, Peak Steel, Ravenscar, Old Peak and Holkham in England, St. Anne's Head, Great Castle Head and the Gann Estuary in Wales, St. Abbs Head in Scotland, Golam Head, Baile na hAbhann and the Aran Islands in Ireland, the Scheldt Estuary in The Netherlands, Ria Ferrol in Spain, the Canary Islands, Madeira, the Cape Verde Islands, and the Azores (the last four together are referred to as Macaronesia) (Fig. 1). However, whenever appropriate, material from other regions (in and outside Europe) was also included to test specific hypotheses, to provide phylogenetic reference taxa (e.g. outgroups) or to serve as comparative test material during the development and evaluation of analytical methods.

In order to screen, quantify and analyze biological diversity in *Littorina*, a wide array of techniques was implemented by the AMBIOS consortium. Phenotypic variation was assessed with morphometric analyses of shell features (calipers measurements and computer-aided image analysis) and thermal tolerance experiments. Genotypic variation was assessed by protein electrophoresis and a number of DNA techniques. The former included starch gel electrophoresis (SGE), polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF) and two-dimensional electrophoresis (2DE) of allozymes, esterases (EST), radular myoglobins (Mb) and general proteins (GP). The DNA techniques involved Random Amplified Polymorphic DNA (RAPD) fingerprinting, restriction site polymorphisms of nuclear genes identified from RAPDs and calmodulin intron sequencing, Single-Strand Conformation Polymorphisms (SSCP) of mitochondrial cytochrome *b* (Cyt*b*), screening microsatellite DNA variation, nucleotide sequencing of calmodulin introns, nuclear rDNAs (18S, 28S, ITS-1) and the mtDNA genes Cyt*b* and cytochrome oxidase I (COI).

Evaluation of some markers and methods used by AMBIOS

INTRODUCTORY REMARKS

Some of the techniques and markers implemented by AMBIOS to measure biodiversity were well-established (e.g. morphometry and allozyme electrophoresis) and therefore did not require an extra assessment of their suitability for the purposes of the project, although refinements were made to the shell measurements used in the morphometric studies and a computer-aided image analysis system had to be developed to allow more variables to be measured and to increase resolution. In contrast, all other analytical methods employed by AMBIOS had to be evaluated with respect to their usefulness for the research program. In some cases techniques had to be adapted for particular applications or even had to be developed from scratch. As these technical developments were one of the aims of AMBIOS, we briefly review in this section our methodological survey and evaluation, and discuss some of the relevant data obtained during the technical survey of the different methods and markers. The further integration of these data (insofar as it is relevant) is provided in the sections on *L. striata* and *L. saxatilis*.

TWO-DIMENSIONAL PROTEIN ELECTROPHORESIS (2DE)

Several protocols for native and denaturing 2DE of radular Mb/GP were tested and compared. However, although they all provided a considerably higher resolution than one-dimensional electrophoretic techniques (e.g. SGE, PAGE and IEF), 2DE appeared to be a less efficient method to screen biodiversity because it entailed several major drawbacks. Firstly, 2DE is very time consuming, taking more than five hours to obtain a single individual profile, and only a few individual profiles could be generated simultaneously. This is because each individual profile requires a complete electrophoretic gel, so that the number of profiles that can be produced at the same time will depend on the number of gels that can be run simultaneously. Secondly, scoring 2D-gels was highly problematic, even when commercial internal markers were used as reference, and gels were screened by densitom-

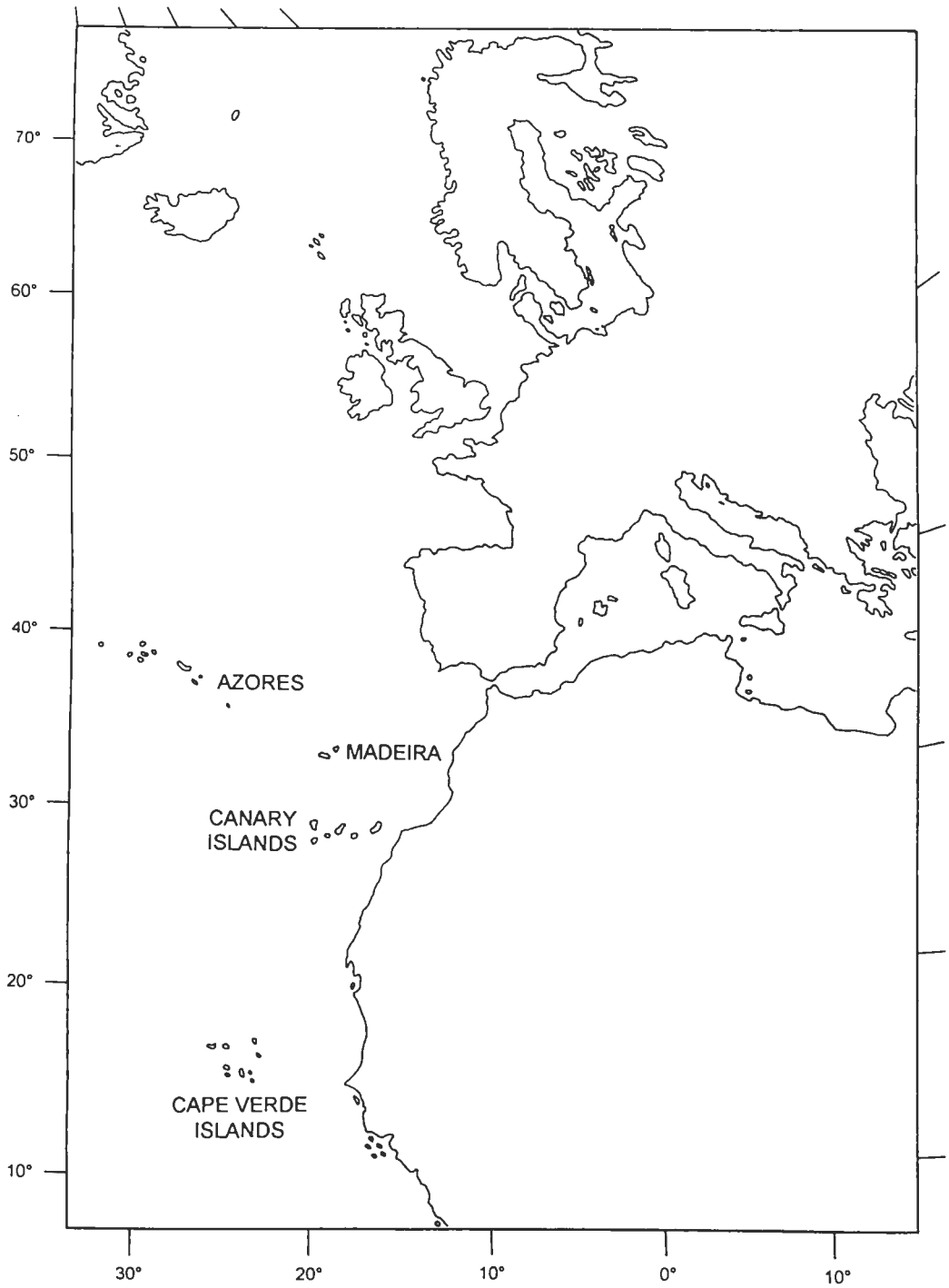

Fig. 1. – Map of the study area covered by the AMBIOS program.

etry and image analysis software. Finally, even when gels were scored appropriately, it remained difficult if not impossible to interpret the spot patterns as genotypes, so that only a phenetic 'spot counting' analysis was possible. Obviously, for a complex, time-consuming and quite costly (e.g. silver-staining, PhastSystem gels) method this is a rather poor amount of information. Hence, 2DE was not further considered as a routine method to survey biodiversity.

Nevertheless, based on a Jaccard's similarity analysis of 60-105 protein spots in over 100 specimens, the 2DE data did provide new evidence suggesting that (1) *L. saxatilis* and *L. arcana* are more closely related to each other than either is to *L. compressa*, (2) *L. compressa* is the genetically least variable species of rough periwinkle, and (3) Azorean *L. saxatilis* is genetically not depauperate or significantly differentiated from British *L. saxatilis*. The closer relationship between *L. saxatilis* and *L. arcana* supports previous studies (e.g. WARD, 1990) (but see BACKELJAU & WARMOES, 1992 and the SSCP work here below), while the reduced genetic variability of *L. compressa* confirms earlier observations by WARD (1990).

RADULAR MYOGLOBINS (Mb) AND GENERAL PROTEINS (GP)

Prior to AMBIOS, only two studies had used Mb in the population genetics and systematics of periwinkles (WIUM-ANDERSEN, 1970; JONES, 1972), and the presence of radular Mb was only known in 17 littorinid species (MEDEIROS *et al.*, 1998). Because of this lack of data and because of the promising nature of WIUM-ANDERSEN's (1970) results, we decided to screen littorinid Mb. Hence, using PAGE and IEF, several aspects of littorinid Mb/GP variation were investigated.

Intraspecific Mb/GP variation was very low in *L. striata*. For example, in 160 specimens there was no detectable Mb/GP variation or differentiation, not even between islands separated by more than 2000 km (Azores vs Cape Verde Islands) (DE WOLF *et al.*, 1998a).

In contrast, *L. littorea* (272 specimens screened) showed a considerable amount of intraspecific Mb/GP variation, particularly in French populations where a higher number of 'unusual' Mb/GP profiles was detected (MEDEIROS *et al.*, 1998). This observation is surprising because *L. littorea* has been reported to show macrogeographic homogeneity over thousands of km (JOHANNESSON, 1992). However, the Mb/GP data support JOHANNESSON's (1992) suggestion that there may be a cryptic taxon in French *L. littorea*. Unfortunately, the Mb/GP variation in *L. littorea* could not be interpreted genotypically and was difficult to quantify.

Nevertheless, a genotypic interpretation and numerical analysis was attempted for microgeographic Mb/GP variation in 142 specimens of *L. obtusata* and *L. fabalis* along a vertical intertidal transect in Galicia (NW Spain) (OLABARRIA *et al.*, 1998). Here, the Mb patterns revealed a high incidence of rare Mb profiles in *L. obtusata* from the *Fucus serratus* zone compared to specimens from the algal belts higher up in the intertidal. Possibly this heterogeneity reflected the marginal situation of *L. obtusata* from the *F. serratus* belt. No comparable differentiation was observed in *L. fabalis*, for which the *F. serratus* zone is a 'normal' habitat. Interestingly, the Mb data could be interpreted genotypically in various ways, but none of these was consistent for all littorinids investigated (including the data from the literature). Moreover, the 'traditional' picture of a dimeric Mb controlled by a single locus (e.g. READ, 1968; TERWILLIGER & READ, 1969) could not be confirmed. However, assuming that two loci code for a monomeric (or dissociated dimeric) Mb a data set was produced for the flat periwinkles in which no significant deviations from Hardy-Weinberg expectations were detected. However, this interpretation could not account for all available littorinid Mb data (OLABARRIA *et al.*, 1998).

The Mb patterns also provided taxonomic and phylogenetic data, even though the Mb profiles did not distinguish consistently between highly similar and closely related species such as *L. obtusata* and *L. fabalis* or *L. saxatilis*, *L. compressa* and *L. arcana*. For this latter group the Mb patterns initially appeared to separate *L. arcana* from both other species (MEDEIROS *et al.*, 1998), but a more extensive analysis has shown that this is not the case and that *L. arcana* and *L. saxatilis* are polymorphic for the same Mb variants. Despite this lack of resolution at the level of closely related species, Mb profiles yielded interesting results at supraspecific lev-

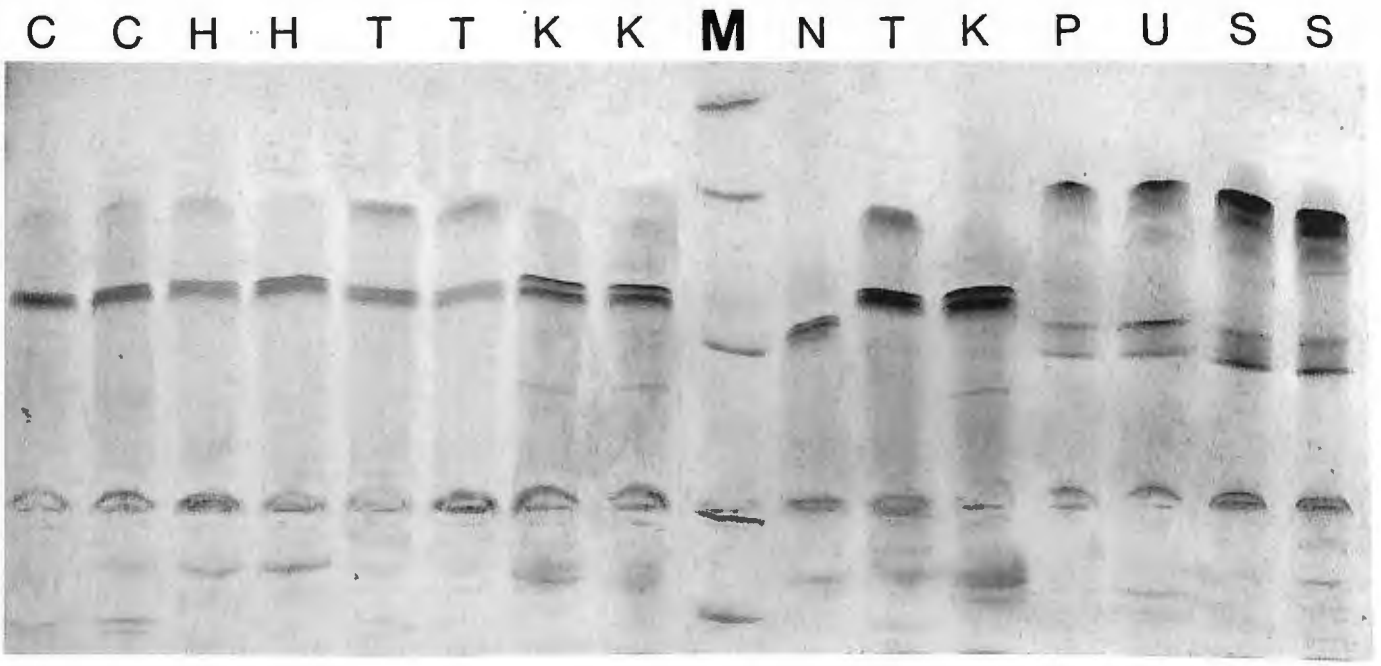

Fig. 2. – IEF profiles of radular Mb (and general proteins) in a pH gradient of 4-6.5 (with pH 4.0 at upper gel side), stained with Coomassie Brilliant Blue. Abbreviations: C: *Cenchritis muricatus*, H: *Nodilittorina hawaiiensis*, K: *L. keenae*, M: pH marker, N: *Melarhaphe neritoides*, P: *L. plena*, S: *L. saxatilis*, T: *L. striata*, U: *L. scutulata*.

els. Using IEF, *M. neritoides* had a unique pattern shared by no other littorinids investigated (Fig. 2), yet with PAGE, the Mb profile of *M. neritoides* was indistinguishable from that of *L. striata* (MEDEIROS *et al.*, 1998). Furthermore, the Mb pattern (both PAGE and IEF) of this latter species seemed to be conservative as it was shared with, or very similar to, those of the species from the genera *Nodilittorina*, *Littoraria* and *Cenchritis* (Fig. 2). Within the genus *Littorina*, the Mb pattern of *L. striata* also resembled very strongly that of *L. keenae* (Fig. 2), which is considered as the next basal species in the genus. Interestingly, *L. scutulata* and *L. plena*, had a distinct Mb pattern that differentiated both species from *L. striata* and *L. keenae* on the one hand and all remaining *Littorina* species, on the other (Fig. 2). This latter group of *Littorina* species had a *L. saxatilis*-like Mb pattern, except for the two flat periwinkles, which had a distinct Mb profile (MEDEIROS *et al.*, 1998). These results are partially at variance with current taxonomic groupings, even though they are in line with a basal position for *L. striata* and *L. keenae*, as well as with a possible taxonomic grouping of *L. scutulata* and *L. plena*. The data for the 'higher' *Littorina* species may have been confounded by ecophysiological and/or phylogenetic convergence (MEDEIROS *et al.*, 1998).

Altogether, the Mb/GP results suggest that, even if the genetic background of these markers in littorinids is still controversial, they may provide an interesting tool for phenetic analyses of variation, as well as for ecophysiological and phylogenetic purposes. Therefore we suggest that littorinid Mb variation is certainly worth further investigation.

NON-SPECIFIC ESTERASES (EST)

Non-specific EST have been used with variable success in several littorinids of interest to AMBIOS (e.g. VUILLEUMIER & MATTEO, 1972; BERGER *et al.*, 1975; NEWKIRK & DOYLE, 1979; MILL & GRAHAMME, 1988; DYTHAM *et al.*, 1992; MILL & GRAHAME, 1992; MAZON *et al.*, 1998). In the present context we assessed the usefulness of EST in three case studies based on IEF.

A first study of 142 specimens of flat periwinkles by OLABARRIA *et al.* (1998) showed that (1) EST profiles consistently distinguished between the two flat periwinkles in Galicia, (2) the mean numbers of individual EST bands (as an indirect measure of heterozygosity) were significantly higher in *L. obtusata* than in *L. fabalis*, and (3) *L. fabalis* was more heterogeneous than *L. obtusata*. Neither species showed significant EST differentiation between specimens from different intertidal algal levels or sexes. These observations largely agree with previous studies, suggesting that *L. obtusata* tends to be more heterozygous, but less heterogeneous than *L. fabalis* (e.g. JANSON, 1987; BACKELJAU & WARMOES, 1992; ZASLAVSKAYA *et al.*, 1992; ROLAN-ALVAREZ *et al.*, 1995; TATARENKOV, 1995).

In a second study, DE WOLF *et al.* (1998a) found that individual EST variation in 710 specimens of *L. striata* was very high, but unrelated to sex, shell morphology, or wave-exposure regimes. Even at macrogeographic scales (i.e. Azores vs Cape Verde Islands) there was no consistent pattern of EST differentiation, a conclusion that confirms parallel allozyme work by DE WOLF *et al.* (1998b).

Finally, the third investigation focused on EST variation in 637 specimens of *L. littorea*. This work supported the previous Mb data suggesting that French populations are differentiated from other European *L. littorea*, and thus may represent a cryptic taxon. More surprisingly, however, it appeared that in *L. littorea* from the Scheldt Estuary (The Netherlands) numbers of EST bands per individual and interindividual EST band similarities seem to correlate with geography (some degree of isolation by distance), salinity and/or heavy metal pollution.

Clearly, these three studies indicate that the IEF analysis of EST variation is an easy, fast and useful technique in biodiversity studies, even though the genetic interpretation of EST variation is usually very difficult. We would therefore qualify it as a 'quick and dirty' method that must only be used in conjunction with other techniques

DNA EXTRACTION PROCEDURES

Within the AMBIOS program several DNA techniques have been developed and implemented. This required, in the first place, suitable DNA extraction protocols. In this respect, molluscs often pose severe problems because they secrete large quantities of mucopolysaccharides (GRENON & WALKER, 1980) and polyphenolic proteins (RZEPECKI *et al.*, 1991), which copurify with DNA and interfere with the enzymatic processing of nucleic acids (e.g. CHAPMAN & BROWN, 1990; SHIVJI *et al.*, 1992; RUMPHO *et al.*, 1994). Several DNA extraction protocols have been developed to overcome these problems (e.g. JIANG *et al.*, 1997; CHASE *et al.*, 1998; MIKHAILOVA & JOHANNESSON, 1998), but we routinely applied the procedures of ASHBURNER (1989) or WINNEPENNINCKX *et al.* (1993) (adapted so that tissues were not ground under liquid nitrogen, but instead were minced using a scalpel). Yet, within the context of AMBIOS we also used faster (i.e. single-step), easier and less expensive DNA extraction protocols, one of which was particularly well-suited to handle individual periwinkle embryos (SIMPSON *et al.*, 1999).

The procedure of SIMPSON *et al.* (1999) was as follows: individual embryos (0.4-0.7 mm) were placed in a 0.5 ml microcentrifuge tube with 10 µl extraction buffer (50 mM KCl, 10 mM Tris HCl at pH 8.3, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.9% Tween-20 and 10 mg/ml Proteinase K). Prior to incubation, mineral oil (20 µl) was added to each tube. Samples were then incubated for 60 min at 65°C, and subsequently denaturated for 15 min at 94°C. The extracted samples were either used directly for PCR amplification or were kept frozen at -20°C for up to five months. The buffer can be made in advance and stored at -20°C indefinitely, although Proteinase K must be added fresh before use.

For the SSCP analyses, the DNA extraction protocol of SMALL *et al.* (1998) was used. This protocol is not only fast

(it takes 10 min instead of several hours), but can also be performed on the bench rather than in a fume hood. Approximately 0.1 g of tissue was incubated in 225 µl extraction buffer [0.1% Tween-20, 5% Chelex resin (Bio-Rad, Richmond, CA, USA)] for 15 min at 95°C, and extracts were agitated after 7 min. After settling, the extracts were stored at -20°C until PCR-amplification.

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

Because of its speed, ease and relatively low cost, RAPD has become a popular DNA technique in population genetics (e.g. HADRYS *et al.*, 1992; BOWDITCH *et al.*, 1993). However, the method has a number of considerable limitations (e.g. the dominant/recessive nature of genotypic band profiles) and presents several interpretative and/or analytical caveats (e.g. BACKELJAU *et al.*, 1995b and references therein). Therefore an assessment of its usefulness in the context of AMBIOS was necessary. This was done in two specific studies.

DE WOLF *et al.* (1998b) surveyed RAPD variation in 320 specimens of *L. striata* from five populations (Azores, Canary Islands and Cape Verde Islands) using two RAPD primers (5'-ACGCGCATGT-3' and 5'-TCACGTCCAC-3') yielding six consistently scorable, polymorphic bands (i.e. loci). Since parallel allozyme studies on four to five enzyme loci in 66 populations of the same species revealed no consistent deviations from Hardy-Weinberg equilibrium conditions, it appeared reasonable to assume that the RAPD polymorphisms would also conform to Hardy-Weinberg expectations. Hence, using the statistical framework of LYNCH & MILLIGAN (1994), the RAPD data were analyzed as classical dominant/recessive di-allelic loci. This yielded heterozygosity and F_{st} estimates that were highly congruent with those obtained from allozyme data. Moreover, just as with the allozyme data, the RAPD analyses suggested that northern populations (Azores) were less heterozygous than southern ones (Cape Verde Islands). Obviously, this congruence between allozymes and RAPD warranted the further use of the latter.

In the other study, involving RAPD variation in *L. saxatilis* it appeared that primer RAPD-H (5'-GCCGTGGTTA-3') amplified a 700 bp band in form M but not in H (GRAHAME *et al.*, 1997). This prompted us to further characterize this 'diagnostic' RAPD band by band-stabbing and using the band stab as template for a second round PCR. The resulting band was cloned and, after hybridisation analysis, seven different sequences were detected in 24 clones examined. Eighteen of the clones yielded one of two different sequences. One additional sequence was found in two clones and the other four sequences were only observed in single clones. One of these latter contained a microsatellite-like region which was studied in some detail (see 'Microsatellite DNA'). When specific primers were designed to the other sequences identified from RAPD-H, various problems were encountered (e.g. inconsistent amplification, confounding length variation, incorrect priming, etc.) and hence they were inappropriate for further analysis.

In an analogous way, primer RAPD-X amplified a band which initially appeared to be diagnostic for *L. saxatilis* M. The band was similar in size to a band shared by both the M and H form. Therefore this band region was band stabbed in a representative of each form and the amplification products cloned. A range of PCR product sizes was found from each. Twelve amplification products were cloned from form H and 14 from form M. None of the products appeared to be useful markers, except for one which showed a variable *Msp*I restriction site (clone X80) (see 'Restriction site analysis').

Obviously, these results show that, although RAPD may be complementary to allozyme data, care must be taken when relying on RAPD bands to 'diagnose' taxa. Morover, the fact that we have shown that single RAPD bands may contain several very different sequences supports previous concerns on the usefulness of RAPDs as phylogenetic (and to a lesser extent population genetic) markers (BACKELJAU *et al.*, 1995b and references therein). Nevertheless, given the large amount of data that can be generated with RAPD, we consider this technique as a convenient 'first step' to uncover hidden variation, but without further analyses as described above, it remains a 'quick and dirty' method, which is best used in conjunction with other molecular markers.

SINGLE-STRAND CONFORMATION POLYMORPHISMS (SSCP)

The methodological rationale behind SSCP (and related techniques) is well-explained by DEAN & MILLIGAN (1998). Although this relatively new method (ORITA *et al.*, 1989, HAYASHI, 1991; FUJITA & SILVER, 1994; KIM *et al.*, 1995) has much to offer to molecular systematics and population genetics (e.g. HISS *et al.*, 1994; BLACK & DUTEAU, 1997; ORTI *et al.*, 1997), it has hitherto only been applied in one molecular genetic study of marine gastropods (KYLE & BOULDING, 1998). Hence, by adapting and applying SSCP, the AMBIOS project made a fundamental contribution to the wider use of this technique. To this end we developed a simple, non-radioactive SSCP method using a new nucleic acid stain, SYBR GOLD(R) (Molecular Probes, Eugene, Oregon, USA). Moreover, rather than using a DNA sequencing apparatus, we performed SSCP separations on a simple protein electrophoresis apparatus.

The protocol was as follows. After DNA extraction using the Chelex protocol described above (SMALL *et al.*, 1998), DNA was PCR-amplified for Cyt*b* fragments iii (212 bp) and iv (225 bp) with the primers:
iii F 5'-GGGCTTCTTACCAAAACCAACACAC-3',
iii R 5'-GAGGGTAGCGTTGTCAACTGC-3',
iv F 5'-GCAGTTGACAATGCTACCCTC-3' and
iv R 5'-GGACTAGGGCCGAAAGTATAAATAAAAGG-3'.
PCR was performed in 20 µl reaction volumes containing 1.5 µM of each primer, 80 µM of each dNTP and 0.5 µl crude DNA extract (SMALL *et al.*, 1998). PCR cycles were preceded by a denaturation incubation for 3 min at 94°C, then held at 80°C while adding 0.5 U of DNA polymerase. Thirty cycles followed: 94°C for 30 s, 45°C for 45 s and 72°C for 30 s, with a final 2 min at 72°C. SSCP samples were then pre-

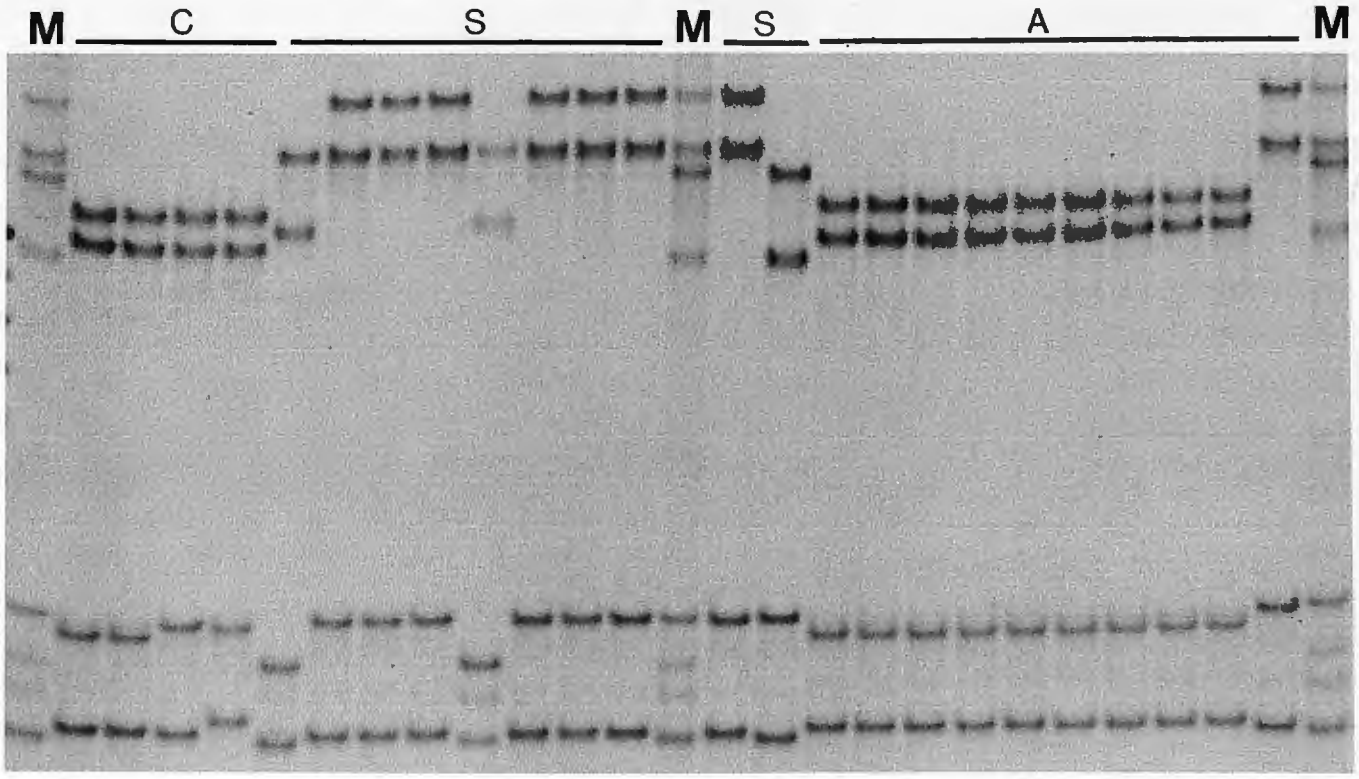

Fig. 3. – SSCP profiles of variation in two fragments of Cyt*b* in *L. compressa* (C), *L. saxatilis* (S) and *L. arcana* (A). Allele marker (M).

pared by mixing 2 µl PCR product with 22 µl denaturation buffer (95% formamide, 20 mM EDTA, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol). The SSCP samples were denatured at 94°C for 5 min, then placed in ice cold water, and 6 µl was loaded into a well in a precooled 160 x 220 x 1.5 mm vertical 9% polyacrylamide gel (37.5:1 acrylamide to bisacrylamide) with 0.75 x TAE (SAMBROOK *et al.*, 1989) in both the gel and the buffer, and with 200 ml buffer in the bottom chamber and 400 ml buffer in the top chamber of a Fisons FEC 175 Adjustable Vertical Gel System. Gels were run for 16 hours at 300 V and 7°C. Staining was done with 0.5 x SYBR GOLD in water for 15 min and illumination under UV light. A 100 bp ladder was included in each run as was an allele standard constructed from the common alleles. These allele standards and all subsequently discovered mobility variants were sequenced.

During the developmental phase of this SSCP protocol it appeared that electrophoretic mobilities differed profoundly depending on whether 0.5x or 1x TAE was used. Furthermore, mobilities varied with buffer volume, temperature, voltage, acrylamide to bisacrylamide ratio and gel or buffer additives such as Mg^{++}, Tween or KCl. Clearly, SSCP analyses do require a stringent standardisation of the protocol. The conditions described here were best for running the two Cyt*b* fragments on the same gel.

The usefulness of SSCP analyses in the context of AMBIOS was demonstrated with three case studies involving Cyt*b*. The first analysis assessed the genetic structure and relationships in 586 specimens of the three rough periwinkles, *L. saxatilis*, *L. arcana* and *L. compressa* from Ireland and the UK (Fig. 3). A total of 38 haplotypes were detected, several of which (including the most common one) were shared by *L. arcana* and *L. compressa*, but were absent or very rare in *L. saxatilis*. In contrast, the most common haplotype in *L. saxatilis* was rare in both other species (Fig. 3). *L. compressa* and *L. arcana* were genetically more similar to each other than each of them was to *L. saxatilis*. At the population genetic level, the three species showed similar geographic structuring, in that northern English populations were differentiated from populations from Cornwall and Ireland (SMALL & GOSLING, 2000).

The two other SSCP case studies dealt with the population genetics and species status of *L. saxatilis*, *L. neglecta* and *L. tenebrosa*. A total of 1537 specimens from Ireland and the UK were investigated. Genetic diversity varied among regions, with the populations from western Ireland, northeastern England and southwestern England tending to have higher diversity than those from elsewhere. Neither *L. tenebrosa* nor *L. neglecta* showed unique haplotypes. In addition, both analyses revealed high amounts of gene flow between the three taxa, and an analysis of molecular variance (AMOVA: see EXCOFFIER *et al.*, 1992) showed that, particularly for *L. tenebrosa*, geographic distance explained nearly twice as much of the variance as habitat type. Hence gene flow was more restricted by distance than by habitat type. Similarly it appeared that exposure regime had little effect on gene flow at most sites. All this suggests that the three taxa

are conspecific, and that *L. neglecta* and *L. tenebrosa* should be considered as ecotypes of *L. saxatilis*.

These data prove that SSCP analyses are an efficient and powerful tool to investigate biodiversity. Particulary, the possibility of screening large numbers of specimens for genotypic DNA variation and of limiting sequence efforts to only those variants that appear to be different, makes the techique highly informative, since it gives data on both the genotypes and the nature of the alleles or haplotypes. Moreover, although not applied in the AMBIOS work, SSCP of nuclear genes allow hetero- and homozygous individuals to be distinguished, thus enabling classical population genetic assessments.

CALMODULIN VARIATION

Because the analysis of calmodulin introns is considered to be a useful tool for surveying neutral DNA polymorphisms (CORTE-REAL *et al.*, 1994; PALUMBI & BAKER, 1994, 1996), it was decided to test these markers in the context of AMBIOS.

The universal calmodulin primers of CORTE-REAL *et al.* (1994) were used to amplify calmodulin introns in *L. littorea*, *L. saxatilis* (forms H and M), *L. sitkana*, *L. natica*, *L. aleutica*, *L. subrotundata*, *L. compressa*, *L. arcana*, *L. obtusata*, *L. fabalis* and *Melarhaphe neritoides*. The PCR reaction mixture (50 µl) contained 50 mM KCl, 10 mM Tris HCl pH 9.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% (w/v) gelatin, 200 µM of each dNTP, 25 pmol of each primer, 25 ng DNA and 1U DNA polymerase. The PCR conditions were: 1 x (5 min 94°C), 35 x (1 min 94°C, 1 min 52°C, 2 min 72°C), 1 x (5 min 72°C). Amplification products were run on a 1% agarose gel containing 0.5 nh/µl ethidium bromide.

Amplification products of four species (*M. neritoides*, *L. littorea*, *L. aleutica* and *L. saxatilis*) were cloned and sequenced for further characterisation. Three separate introns (CAL-1, CAL-2 and CAL-3) were recognised, the sequences of which displayed little apparent homology. This suggests that they may be introns of three different calmodulin genes. Both CAL-1 and CAL-2 occurred in all *Littorina* species studied, while CAL-3 was only found in *L. littorea* and *L. aleutica*. *M. neritoides* had two introns (CAL-S and CAL-L), but their homology with those of *Littorina* could not be established.

Although the sequences of CAL-1 and CAL-2 were variable within the rough periwinkles, they showed no fixed differences that consistently separated four forms of *L. saxatilis* (H, M, *L. tenebrosa* and *L. neglecta*), or that distinguished *L. saxatilis* from the two other rough periwinkles, *L. arcana* and *L. compressa*. However, the flat periwinkles differed markedly from the rough periwinkles, suggesting that the calmodulin introns may provide a phylogenetic signal to resolve relationships among more distantly related species. The fact that *L. littorea* and *L. aleutica* contain a third intron is also suggestive in this context, because these two species are separated on the consensus tree of REID (1996) by a polychotomy of three branches, two of which were represented by *L. subrotundata* and *L. sitkana*, which are species that do not seem to have the third intron.

The population genetic usefulness of the CAL sequences was investigated by searching for polymorphic restriction sites. This yielded one variable restriction site (for *Taq*I) in CAL-1 (Fig. 4), due to an indel between positions 362-366. During the restriction digest screening of this locus, a third allele was found, the exact sequence basis of which remains to be investigated. Another variable restriction site was found in CAL-2

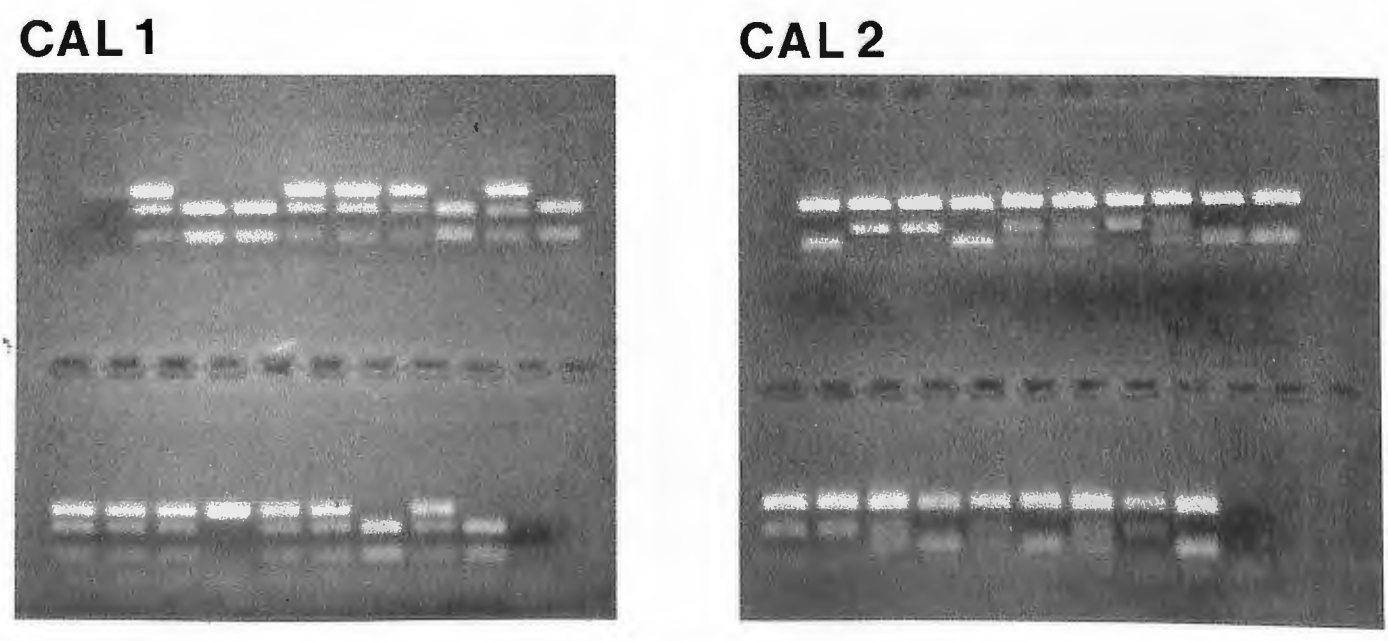

Fig. 4. – Example gels of restriction fragment variation in CAL-1 (restriction site obtained with *Taq*I) and CAL-2 (restriction site obtained with *Dde*I) in *L. saxatilis*.

(for *Dde*I) (Fig. 4). This polymorphic site yielded four alleles. The restriction analysis of these two calmodulin intron loci, combined with similar work on two restriction loci derived from apparently diagnostic RAPD bands, will be discussed under the heading 'Restriction site analysis'.

Finally, CAL-1 also contained a variable microsatellite-like region, whose usefulness will be discussed under the heading 'Microsatellite DNA'.

In view of these results we think that a further characterization and survey of littorinid calmodulin intron variation will be particularly useful for resolving supraspecific phylogenetic problems, but is probably less relevant for studying low level taxonomic problems.

MICROSATELLITE DNA

With less than 15 published papers worldwide, gastropod mini- and microsatellite analysis is currently still in its infancy. Although some work has been done on abalone minisatellites (HUANG *et al.*, 1997; MUCHMORE *et al.*, 1998), there is, to our knowledge, no published information available on microsatellites of marine gastropods. This is surprising as microsatellites are often highly polymorphic markers, which are increasingly becoming popular in (population) genetic analyses (e.g. JARNE & LAGODA, 1996; GOLDSTEIN & SCHLÖTTERER, 1999). Therefore AMBIOS aimed at bridging this gap by developing microsatellites for periwinkles and, as a consequence, producing the very first published data on prosobranch microsatellites (WINNEPENNINCKX & BACKELJAU, 1998a, b).

The protocols for the development and routine screening of five microsatellite markers in *L. striata* were described by WINNEPENNINCKX & BACKELJAU (1998b). During the developmental phase of the work, it became apparent that the quality of the microsatellite profiles strongly depended on the performance of the DNA polymerase during PCR amplification, the PCR conditions [i.e. the concentrations of bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), and magnesium sulfate ($MgSO_4$)] and on the type of radioactive labeling used (WINNEPENNINCKX & BACKELJAU, 1998a). For example, [α-^{35}S]dATP labeling during PCR amplification produced considerably more artificial 'stutter' bands than end labeling one of the flanking primers with [γ-^{32}P]dATP. Moreover, with this latter type of labeling all DNA polymerases tested performed well. In contrast, some DNA polymerases yielded poor amplifications when incorporating [α-^{35}S]dATP. In addition, the performance of some DNA polymerases was negatively affected by adding DMSO (but see SHEN & HOHN, 1992), whereas adding BSA or $MgSO_4$ appeared to have no effects and certainly did not improve the quality of the microsatellite patterns, as is usually claimed (e.g. ROUX, 1995; KREADER, 1996).

Five microsatellite loci were characterized in 16-35 specimens of *L. striata* from several localities. The five loci showed a high amount of allelic polymorphism, with at least 15 to 27 alleles/locus (average 19.2 alleles/locus). A subsequent population survey showed that the numbers of alleles were even higher, with up to 36 alleles for locus Lstri1.151 in São Miguel. The five loci showed large heterozygote deficiencies, and an overall exact test for Hardy-Weinberg equilibrium, under H_1 = heterozygote deficit, indicated significant heterozygote deficits at all loci in the three populations surveyed [Azores: Flores (n = 32) and São Miguel (n = 40); Cape Verde Islands: Sal (n = 12)]. As scoring the gels was straightforward, and since allozyme analyses of four to five loci in 66 populations of *L. striata* showed no significant heterozygote deviations, we suspect that the consistent underscoring of heterozygotes with the microsatellites is due to a high prevalence of null alleles (possibly in combination with too small sample sizes compared to the large numbers of alleles and possible genotypic combinations). The presumed occurrence of null alleles is supported by the observation that all loci revealed different non-amplifying samples, suggesting that lack of amplification was not due to particular individuals (e.g. because of poor quality of their DNA). Unfortunately it was beyond the scope of AMBIOS to perform breeding experiments to test the inheritance of the observed microsatellite variation. Anyway, the alleged high frequency of null alleles (in the Sal population they reached frequencies of up to 0.48) imposed severe limitations on the usefulness of these microsatellites. This was even further exacerbated by the lack of cross-species amplification of the *L. striata* microsatellites in *Nodilittorina punctata*, *L. obtusata*, *L. saxatilis* and *L. arcana*.

Apart from the deliberate search for microsatellites in *L. striata*, we uncovered microsatellites serendipitously in the calmodulin intron CAL-1 and in a RAPD-band obtained with primer RAPD-H (5'-GCCGTGGTTA-3') in *L. saxatilis* M (see also 'Calmodulin variation', 'Random amplified polymorphic DNA (RAPD)' and 'Restriction site analysis').

The CAL-1 intron in *L. saxatilis* contained a complex microsatellite-like region with a variety of dinucleotide and trinucleotide repeat motifs responsible for a presumably allelic length variation. Hitherto, this microsatellite-like sequence was determined in two specimens of *L. saxatilis*, yielding respectively $(GTCT)_2(GTTT)_2(GT)_3GA(GT)_2GA(GT)_{18}TT(GT)_5(GC)_4(GT)_{10}TTGTGA(GT)_{11}(GTCT)_7GAGTC(TG)_2AGTTAA$ and $(GTTT)_2(GT)_3GA(GT)_2GA(GT)_{18}GA(GT)_4(GC)_2(GT)_{27}(CTGT)_5GAGTTAA$. The complex structure of this repeat region suggested that it would be of only limited usefulness for microsatellite analysis.

A more useful microsatellite was found in a RAPD-H (5'-GCCGTGGTTA-3') band amplified in *L. saxatilis* M (GRAHAME *et al.*, 1997). This microsatellite involved a GTT repeat motif which was often perfect, even though in some specimens it showed a variety of interruptions attributable to point mutations and single base pair deletions. The primers to amplify the repeat were RAPDRPT1 (5'-TTTACCTCATCTGTTGCGTCC-3') and RAPDRPT2 (5'-AATAACATACAGCGACGACGG-3'). Amplifications with these primers produced one or two bands in an approximate size range of 95-530 bp, with nearly no alleles in the size range 300-400 bp. Although some alleles >400 bp represented amplification products of unclear nature, they were mostly good GTT repeats. Fortunately, the vast majority of

alleles had a size <300 bp. A total of 698 specimens of *L. saxatilis* H and M from eight localities in Ireland and the UK were surveyed for approximate length variation at this microsatellite locus. This suggested that, on average, the amplification products from *L. saxatilis* H are larger than those from *L. saxatilis* M. Because alleles were not exactly sized, precise genotype frequencies were not available. Nevertheless, conformance with Hardy-Weinberg expectations was tested on the basis of alleles 'binned' into 5 bp classes. Each 'binned' class was then considered as an 'allele'. Genotype frequencies based on these 'alleles' showed significant deviations from Hardy-Weinberg conditions in all populations, except for *L. saxatilis* M at one site in SE England (Folkestone). In all cases these deviations were due to serious heterozygote deficiencies, the cause of which is still unclear (null alleles?). Anyway, despite this possible flaw, and assuming neutrality of the repeat motif (and no linkage to loci under selection), the differential size distribution of the alleles in *L. saxatilis* H and M may suggest some sort of genetic divergence between the two forms.

In conclusion, despite the current popularity of microsatellites, we found the development and application of these markers in the context of AMBIOS too cumbersome, time-consuming, costly and unreliable (cf. the high prevalence of null alleles) to merit further pursuit.

RESTRICTION SITE ANALYSIS

The application of restriction site analyses is widespread and very popular in population genetics (e.g. AVISE, 1994), so that it was felt superfluous to provide a technical evaluation of the method. Nevertheless, by way of experiment, a restriction site analysis of the *L. saxatilis* complex was performed on four nuclear loci. The primers
CAD1F, 5'-TGCACATCATCATGCCAAA-3' and
CAD1RB, 5'-TCTTCAGAGCAGGGTTCCATT-3'
were used to amplify the intron locus CAL-1, and
CAD2F, 5'-CTGCAGATGGTGACGCAA-3' and
CAD2R, 5'-CTGACGGTGAGTGACAATCG-3'
were used to amplify CAL-2 (see 'Calmodulin variation').

Two polymorphic loci obtained from cloning and sequencing apparently diagnostic RAPD bands (GRAHAME *et al.*, 1997) were also screened. One of these (X-80) was identified from sequences generated with the RAPD-X primer (OPY-01, 5'-GTGGCATCTC-3') and was amplified with the primers X80a 5'CATCTCTGTGTTGAAAGAGGGT-3' and X80b 5'-CAGAACTAAACTGAAGAAACCCG-3'.

The other RAPD-locus (referred to as DELETION) was derived from RAPD-H band containing the microsatellite repeat motif described under the heading 'Microsatellite DNA'. The flanking sequence of this GTT repeat indeed revealed a deletion useful for a restriction site analysis, in which the locus was amplified with the primers RAPDRPT3, 5'-AGTAACGGCAGACGCCAT-3' and RAPDRPT4, 5'-TTATTCTTGTCTTCTCTGCC-3'.

All PCR amplifications were performed in 25 µl reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 200 µM of each dNTP, 12.5 pmole of each primer, 12.5 ng DNA and 0.5 U DNA polymerase. PCR cycling conditions were: 1 x (5

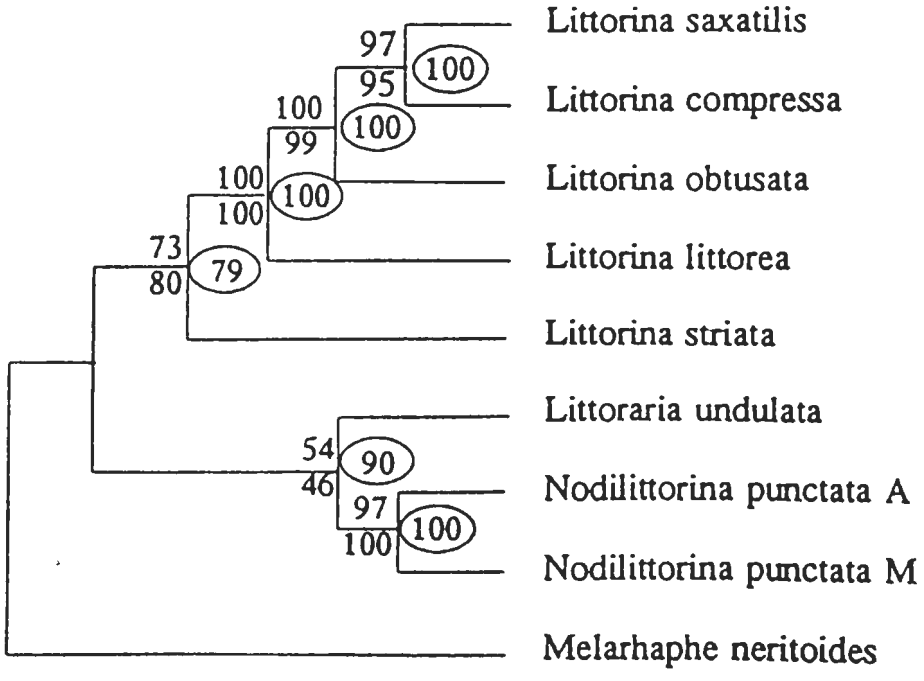

Fig. 5. – Maximum Parsimony (with successive weighting) and Maximum Likelihood tree of littorinid relationships based on 18S rDNA sequences. Values at the branching points are bootstrap or support values obtained with fastDNAml (below), PUZZLE (encircled) and Maximum Parsimony (above). A: Atlantic; M: Mediterranean.

min 94°C), 35 x (1min 94°C, 30 s 55°C, 1 min 72°C) and 1 x (5 min 72°C), except for CAL-1 in which the annealing temperature was 57°C and DELETION in which annealing was at 57°C and the extension time 30 s.

The allelic variations in CAL-1, CAL-2 (Fig. 4) and X-80 were screened with the restriction enzymes *Taq*I, *Dde*I and *Msp*I respectively, and the restriction digests were separated on 2% agarose gels. The alleles at the DELETION locus were detected by their size difference after electrophoresis of the PCR amplification product in a 3% agarose gel.

The genotypic profiles obtained were analyzed as allozyme or microsatellite data. As such, the allele frequency data at the four loci were used to derive phylogenetic relationships between *L. arcana*, *L. compressa*, *L. saxatilis*, *L. neglecta* and *L. tenebrosa* (in total 789 specimens from 32 locations in the UK, Ireland and Sweden). The resulting dendrograms clustered populations more or less according to the three well-recognized species *L. saxatilis*, *L. arcana* and *L. compressa*, with the former two as sister taxa. However, never were all populations placed correctly at the same time and no restriction patterns appeared to be diagnostic for any of the taxa included.

After correcting for multiple testing, only three out of 128 tests showed significant deviations of Hardy-Weinberg equilibrium conditions and there was no sign of linkage in the data. However, the population genetic analysis of the four loci provided little new information that was not already obvious from allozyme and SSCP data. This fact, together with the unstable phylogeny produced by the restriction site analysis, meant that this method was considered less useful for AMBIOS purposes, particularly as the SSCP analyses allowed a much higher resolution.

NUCLEAR RIBOSOMAL AND MITOCHONDRIAL DNA SEQUENCING

Apart from the application of Cyt*b* sequences in the SSCP analyses, DNA sequencing was also used on its own, mainly to infer phylogenetic relationships. Evidently, from a technical point of view DNA sequencing did not require an evaluation.

Nuclear rDNA sequences were used to investigate the phylogenetic position of *L. striata* and to test its suitability as a marker to resolve recent divergences that took place less than 40 Myr ago (WINNEPENNINCKX *et al.*, 1998). This was tested by comparing complete 18S rDNA sequences (±1800 bp) in nine littorinids (*Nodilittorina punctata* from the Mediterranean and the Cape Verde Islands, and *Melarhaphe neritoides*, *Littoraria undulata*, *L. striata*, *L. littorea*, *L. obtusata*, *L. compressa* and *L. saxatilis*). Using distance matrix, parsimony and maximum likelihood methods, these sequence data placed *L. striata* consistently at the base of the *Littorina* clade, although only with the maximum likelihood methods (fastDNAml and PUZZLE) and the successive weighted parsimony analysis did this placement receive bootstrap values >70% (Fig. 5). The topology within the *Littorina* clade completely agreed with current ideas on *Littorina* relationships (REID, 1996). Interestingly, Atlantic

Table 1:
Relative performance of 12S rDNA (12S), 16S rDNA (16S), Cytochrome b (Cyt*b*) and 18S rDNA sequences in resolving relationships between three closely related littorinid species of the subgenus *Neritrema* (after WINNEPENNINCKX *et al.*, 1998).

	12S	16S	Cyt*b*	18S
Number of aligned sites	374	444	651	1841
Parsimony informative sites	9	8	52	11
Bootstrap values for:	2.4	1.8	8.0	0.6
L. compressa + *L. saxatilis*				
NJ (Jukes and Cantor)	73	NA	100	94
MP	NA	NA	100	98
ML	NA	NA	100	96
L. compressa + *L. saxatilis* + *L. obtusata*				
NJ (Jukes and Cantor)	60	100	100	97
MP	56	100	100	93
ML	NA	100	99	96

Table 2:
Heat Coma Temperatures (HCT) and their standard errors (s.e.) in littorinids of the subgenus *Neritrema*.
N: number of specimens examined.

	South Wales		Northeast England	
Species	N	Mean HTC (°C) ± s.e.	N	Mean HTC (°C) ± s.e.
L. saxatilis H	27	31.64 ± 0.34	28	31.96 ± 0.36
L. saxatilis M	30	33.00 ± 0.31	29	31.97 ± 0.20
L. arcana	91	33.13 ± 0.18	28	32.70 ± 0.44
L. compressa	30	34.16 ± 0.43	—	does not occur
L. littorea	21	33.43 ± 0.33	31	31.48 ± 0.30
L. obtusata	21	35.93 ± 0.57	30	32.06 ± 0.68

and Mediterranean *Nodilittorina punctata* showed the same degree of sequence divergence as *L. saxatilis* and *L. compressa*, suggesting that possibly Atlantic and Mediterranean *Nodilittorina punctata* belong to different taxa. Clearly, the 18S data do contain information to resolve more 'rapid' cladogenetic events. In fact, in comparison with previous markers, only Cyt*b* performed better than 18S rDNA in this respect (Table 1) (WINNEPENNINCKX *et al.*, 1998).

In a subsequent analysis of the higher (generic) relationships of the Littorinidae, the performance of 18S rDNA was further compared with that of partial 28S rDNA sequences (31 taxa included, each sequenced for ±1800 bp of 18S rDNA and a 750-800 bp stretch of 28S rDNA). This analysis hitherto suggests that, at this level, 28S rDNA outperforms 18S rDNA, even though both data sets contain a substantial amount of phylogenetic information.

Several attempts to sequence ITS-1 in several littorinids failed despite amplification of this gene posing no problems. Although sequencing was unambiguous as long as the actual rDNA subunit stretches were involved (i.e. the priming regions), it lapsed into apparently overlain sequences after entering the ITS-1 zone. This suggests the presence of different ITS-1 copies, a problem which deserves further attention, but which made ITS-1 unsuitable for the AMBIOS goals.

The relative performance of the mitochondrial Cyt*b* and COI genes was also evaluated. To this end a 537 bp portion of Cyt*b* (of which 2.05% is parsimony informative) and a " 400 bp portion of the COI gene (of which 4% is parsimony informative) were sequenced in a variety of *Littorina* species, including *L. tenebrosa* and *L. neglecta*. It appeared that COI was more variable than Cyt*b* and that COI based phylogenies usually show better bootstrap support than Cyt*b* phylogenies. The Cyt*b* portion here studied did not resolve the phylogeny of the rough periwinkles, was not diagnostic in this species group and did not provide evidence for a species status for *L. neglecta* and *L. tenebrosa*. It did, however, consistently distinguish between the rough and flat periwinkles. In contrast, combined with SSCP analyses, the Cyt*b* provided useful population genetic data (see 'Single strand conformation polymorphisms'). Whereas the COI sequences were more variable than Cyt*b*, they were insufficient to yield well-supported phylogenies. However, the simultaneous use of both data sets produced a stable phylogeny in general agreement with the current, consensus opinion (REID, 1996). A companion restriction fragment analysis of both genes revealed that, although there were RFLP patterns typical of *L. saxatilis*, *L. compressa* and *L. arcana*, there were no truly diagnostic patterns (i.e. occurring in all specimens of a given taxon and never occurring in another taxon). In contrast, there was a degree of haplotype sharing between rough periwinkle species and forms. It is still unclear whether this is due to incomplete lineage sorting of ancestral polymorphisms or to hybridisation events.

In order to design the primers for the previous sequencing work, fragments of *L. saxatilis* mtDNA were cloned and 8022 bp were sequenced, i.e. about half the complete mtDNA of this species. Since this was not a primary goal of AMBIOS, we refer to WILDING *et al.* (1999) for a detailed account of this mtDNA work.

In summary, the 18S and 28S rDNA sequences appear to be very useful for littorinid phylogeny inference at a variety of taxonomic levels. However, the combined mtDNA sequences investigated seem to provide a better resolution of the rough periwinkle relationships. Hence nuclear rDNA and mtDNA sequences seem to complement each other nicely. For population genetic work, DNA sequencing is most relevant if combined with restriction analysis or, better still, SSCP.

ANALYSIS OF THERMAL TOLERANCE

Given that littorinids occur in the intertidal zone, where they may be subject to a wide range of very different thermal loadings, and considering that temperature moderates the effects of almost all environmental and biological factors, it was decided that AMBIOS should consider thermal tolerance as a phenotypic estimate of biodiversity at the physiological level. This could possibly shed light on the functional meaning of other phenotypic traits (e.g. shell morphology, physiology, behaviour) that contribute to biological variation, irrespective of whether these are adaptive or ecophenotypic.

Thermal tolerance was assessed by estimating mean heat coma temperatures (HCT) as defined by MCMAHON (1976), using a method modified from MCMAHON (1990). Acute upper lethal thermal limits (LT_{50} values: estimated temperature of 50% sample mortality) were also measured as described by MCMAHON (1990). Generally, thermal tolerance appeared to be stable within populations, but highly variable between populations and species. All taxa examined (*L. littorea*, *L. obtusata*, *L. fabalis*, *L. compressa*, *L. arcana*, *L. saxatilis* H, *L. saxatilis* M, *L. tenebrosa*, lagoonal *L. saxatilis*, and *L. neglecta*) had HCT values in the range of 30-36°C (Table 2), while acute upper lethal temperatures were about 10°C higher than the HCT values (CLARKE *et al.*, 2000b). There was no clear correlation with shore levels, yet eulittoral fringe species tended to possess higher HCT values than eulittoral species. This is in accord with MCMAHON's (1990) work on tropical littorines. HCT values also varied seasonally and geographically, but showed no correlation with individual size (measured by columella length), except in *L. littorea*, where animals with a columella length >30 mm showed significantly lower thermal tolerances (CLARKE *et al.*, 2000a). Finally, the duration of acclimation in the laboratory had no effect on HCT values, yet higher acclimation temperatures increased HCT values (CLARKE *et al.*, 2000b).

In general, thermal tolerance seemed to be a highly plastic trait showing little if any correlation with the genetic markers investigated by AMBIOS. Moreover, HCT and LT_{50} values do not determine the in situ survival of periwinkles because the tolerance towards a single environmental factor such as temperature may well be greater than the tolerance towards a combination of (interacting) environmental factors (SOUTHWARD, 1958). Hence, although thermal tolerance investigations may provide clues to explain certain patterns of biological variation, they seem less suitable and quite uninformative for assessing genetic and taxonomic components of biodiversity.

Biological variation in a planktonic developing littorinid: The case of *Littorina striata*

L. striata was intensively studied throughout its entire geographic range, which comprises the Macaronesian region, including the Azores, Madeira, the Canary Islands and the Cape Verde archipelago. The species is conchologically polymorphic and two relatively well-defined shell morphotypes can be distinguished: one with a clearly nodulose shell, the other with a 'smooth' shell (REID, 1996, DE WOLF *et al.*, 1997). Both shell types occur microsympatrically, which raises the question as to the ecological, genetic and taxonomic significance of such a polymorphism in a planktonic developing species.

In a first analysis, the spatiotemporal distribution of the two morphotypes was investigated at Ilheu de Vila Franca do Campo (further referred to as 'Ilheu'), a drowned volcanic crater, about 1000 m off the south coast of São Miguel, Azores (DE WOLF *et al.*, 1997). This key site has been extensively described and characterized by FRIAS MARTINS (1976), MORTON (1990), DE WOLF *et al.* (1997) and MORTON *et al.* (1998). The crater consists of two small islands, surrounding a circular lagoon which is connected with the ocean by a small channel and six narrow fissures. As a consequence, the lagoon is extremely sheltered, while the outer margins of the crater are strongly wave-exposed. Hence, environmental conditions differ considerably between the lagoon and the outside, providing an excellent setting to investigate possible ecological correlates of shell polymorphism. Therefore, *L. striata* was collected annually (summers of 1992, 1993 and 1994: these samples were collected prior to the start of AMBIOS) at eight sites at Ilheu. Four sites were located in the lagoon, the other four on the outside of the crater. Of these latter, two were in the splash zone; the other two in the barnacle belt (i.e. midshore). Specimens were separated into nodulose and smooth shelled, and five shell traits were measured with calipers. In total 1078 specimens were included. The statistical analysis of these data showed that (1) nodulose shells are more common in the sheltered lagoon, whereas smooth shells dominate the wave-exposed outside, (2) on average nodulose shells are smaller than smooth ones, (3) regardless of morphotype, shells from the lagoon have a smaller aperture and are less globose than those from the outside, (4) within an exposure regime, smooth specimens have a larger aperture, (5) this morphological patterning remains consistent over time (DE WOLF *et al.*, 1997).

The ecological differentiation between nodulose and smooth *L. striata* was further tested with a translocation experiment at Ilheu (DE WOLF *et al.*, 1997). To this end, 240 specimens of comparable size were collected in the lagoon and on the outside of the crater. Each shell was measured and specimens were then divided into four groups: those transplanted from the lagoon to the outside (n=60), those transplanted within the lagoon (n=60), those transplanted from the outside to the lagoon (n=60) and those transplanted on the outside (n=60). Each specimen was marked with a group-specific colour dot, and animals were recaptured and remeasured every month. The experiment was stopped after five months. It showed that aperture height of specimens transplanted from the lagoon to the outside increased significantly, whereas the other three groups showed no significant morphometric differences between the start and the end of the experiment (DE WOLF *et al.*, 1997).

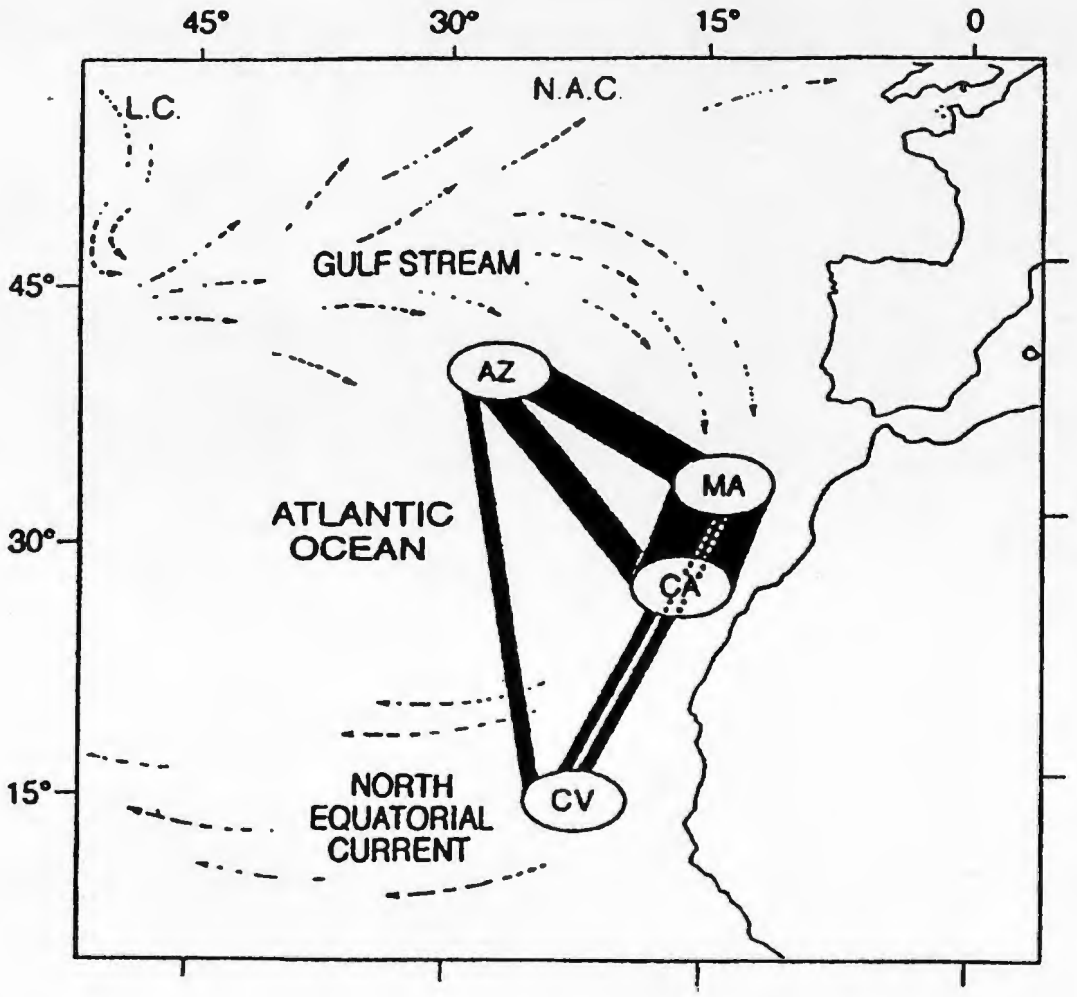

Fig. 6. – Proportional gene flow patterns of *L. striata* in Macaronesia, projected on a map with the trajectories of major surface currents (data from private allele frequencies in five allozyme loci). Abbreviations: AZ: Azores; CA: Canary Islands; CV: Cape Verde Islands; LC: Labrador Current; MA: Madeira; NAC: North Atlantic Current.

An unexpected occasion to evaluate shell polymorphism in *L. striata* occurred in December 1996, when an exceptionally heavy storm hit the Azores (DE WOLF *et al.*, 1999). Immediately after this storm the same sites were sampled at Ilheu as before (1992, 1993 and 1994). The expectation was that, after the storm, wave-exposed specimens should have shifted to a larger aperture size, whereas the sheltered lagoon specimens should have remained largely unaffected. Yet, no clear cut morphological response was observed, since different statistical techniques (i.e. MANOVA + canonical discriminant analysis vs. nested ANOVA) arrived at opposite conclusions. However, the preferred approach, based on a nested ANOVA design contrasting the four years (1992, 1993, 1994 and 1996) with the factor 'year' nested within the factor 'storm', showed no significant storm effect (DE WOLF *et al.*, 1999). Obviously, it remains to be decided whether this lack of response indicates that shell form is not directly related to wave-exposure or whether it is simply due to the possibility that the exposure to increased wave-action during the storm was too brief.

The final analysis of shell variation in *L. striata* tested whether the shell polymorphism in this species showed the latitudinal trend that, as heat stress and predation increase and wave action decreases towards the tropics (e.g. VERMEIJ, 1973, 1978), rocky shore gastropods from lower latitudes tend to have more sculptured (e.g. nodulosity, ...), more globose, shells with smaller apertures and more shell repair marks, than those from higher latitudes (e.g. VERMEIJ, 1978). This model was tested on *L. striata* using 1640 mature specimens from 41 populations covering the entire range of the species (from the Azores, Madeira, the Canary Islands and the Cape Verde archipelago, between 39°41'N and 14°20'N). All shells were measured as before and scored for sculpture (nodulose vs smooth) and the presence of repair marks. The main results of this work were: (1) shell size and weight increased towards the south, (2) there was no consistent pattern in nodulosity between archipelagos, and (3) nodulose shells displayed fewer shell repair marks than smooth shells, but at the same time showed more repair marks in northern populations than in southern ones. The fact that these obser-

vations partly disagree with current ideas on morphological north-south patterns may be due to the unexpected (and indirectly inferred) increased wave action in the Cape Verde islands compared to the three other, more northerly, island groups. This atypical situation may confound the interpretation of morphological patterns in *L. striata*. More generally, it seems that generalizations about macrogeographic shell morphology patterns based on interspecific comparisons are not directly applicable to intraspecific patterns, and may depend strongly on local conditions, which make adequate sampling and data treatment very difficult (DE WOLF *et al.*, 1998d).

The results of the morphometric studies were interpreted as showing that the nonrandom distribution of both shell morphs reflects a functional response to thermal stress and wave exposure. The more globose form, lack of sculpture and larger aperture size (i.e. stronger holdfast on the substrate) protect the smooth specimens better against drag and dislodgement in wave-exposed conditions, where thermal stress is reduced, while the smaller aperture and increased reflectance/convection surface protect the nodulose specimens against the higher thermal stress prevalent in sheltered areas (BRITTON, 1995; DE WOLF *et al.*, 1997, 1998d). The actual underlying mechanism of this functional response remains unknown, even though the translocation experiment at Ilheu tentatively suggests that at least aperture size would be an ecophenotypically plastic trait caused by differential growth rates (DE WOLF *et al.*, 1997).

In order to assess whether the patterns of shell variation in *L. striata* reflect genetic barriers or population structuring, a parallel population genetic survey was performed using allozyme electrophoresis (PAGE), IEF of Mb and EST, RAPDs and microsatellite DNA. A first PAGE analysis of four allozyme loci (*Gpi*, *Pgd*, *Mpi* and *Mdh*) in the 1078 specimens used for the microgeographic shell morphometric survey at Ilheu over the years 1992, 1993 and 1994 (see above; DE WOLF *et al.*, 1997) showed that (1) there were no significant deviations from Hardy-Weinberg equilibrium conditions, (2) both shell forms share a common gene pool, (3) gene flow between populations was high, (4) the population genetic structure remained stable over a sampling period of three years, and (5) genetic and morphological distances between populations were not correlated. Hence, the two shell morphotypes of *L. striata* may be safely considered as being conspecific, implying that their nonrandom spatial patterning persists in the presence of intense gene flow (DE WOLF *et al.*, 1998c). A similar population genetic survey of five enzyme loci (*Gpi*, *Pgd*, *Mpi*, *Mdh* and *Hbdh*) in 41 populations (1640 specimens) derived from the four Macaronesian archipelagos, was performed to complement the macrogeographic morphometric analysis. These allozyme data suggested that, although only very limited amounts of population differentiation were detected, there were indications of a macrogeographic patterning of allozyme variation (Fig. 6), such that at least the populations from the Cape Verde Islands appeared different from those from the other archipelagos because: (1) there were eight unique alleles in the Cape Verde archipelago and two in the Azores (none elsewhere), (2) the mean number of alleles per locus was highest in the Cape Verde Islands, (3) the number of loci revealing between archipelago heterogeneity was highest for comparisons involving the Cape Verde Islands, and (4) both a correspondence analysis and a UPGMA dendrogram separated the Cape Verde Archipelago from the other island groups. In addition, the macrogeographic morphometric study showed that the morphological variation in the Cape Verde Islands was larger than elsewhere (DE WOLF *et al.*, 1998d), while the RAPD and EST data also suggested a higher diversity in this archipelago than elsewhere (DE WOLF *et al.*, 1998a, b). However, there was no significant relationship between geographic and genetic distances. The macrogeographic pattern of variation was tentatively interpreted as being the result of ancient and current oceanic circulations (Fig. 6). *L. striata* probably originated in the Cape Verde Islands since: (1) the oldest known fossils of *L. striata* date from Tertiary deposits in this archipelago (SOWERBY, 1844), (2) these islands are the oldest in Macaronesia (together with Gomera and Fuerteventura in the Canary archipelago) (MITCHELL-THOME, 1976), and (3) both genetic and morphological diversity is highest in the Cape Verde Islands (see above). Given that in the past the Atlantic Ocean was less wide (ADAMS, 1981), while surface currents across the Atlantic may have been stronger than now (SCHELTEMA, 1995, REID, 1996), we suggest that, during the Tertiary, larvae originating from the Cape Verde Islands may have reached the emerging Azores by the westward clockwise circulation, i.e. via the North Equatorial Current from the Cape Verde Islands westward to the Caraibes and the east coast of North America and from there back eastward via the Gulf Stream to the Azores (see GOFAS, 1990). However, in subsequent periods the currents nearer the surface slowed down and the Atlantic widened. Thus larval westward transport from south to north decreased and ultimately became impossible, so that currently the Cape Verde islands may act as a sort of 'cul de sac' for gene flow coming from the north. This tentative scenario could account for the accumulation of higher diversity in the Cape Verde Islands, without spreading this diversity to the other archipelagos.

Biological variation in a non-planktonic developing littorinid: The case of the *Littorina saxatilis* complex

The investigations on the *L. saxatilis* complex focused particularly on (1) the biological interpretation of the forms H, M, *L. tenebrosa* and *L. neglecta* (see Material and Methods), (2) macrogeographic variation in *L. saxatilis*, (3) characterisation of Azorean *L. saxatilis*, and (4) the relationships between *L. saxatilis*, *L. arcana* and *L. compressa*.

For both *L. tenebrosa* and *L. neglecta*, most analyses pointed to the same outcome: neither of these forms seem to represent an independent gene pool or show consistent molecular genetic differentiation from *L. saxatilis*. For example, the restriction analysis of CAL-1, CAL-2, DELETION and X-80, the SCCP analysis of Cyt*b* (SMALL & GOSLING, 2000) and the restriction analysis of COI provided no evidence to support a taxonomic separation of *L. neglecta* and *L. tenebrosa*

at the species level, even though the Cyt*b* RFLP analysis suggested differentiation between *L. saxatilis* and *L. neglecta* on the East coast of England. Furthermore, *L. tenebrosa* and *L. neglecta* do not differ from *L. saxatilis* in heat coma characteristics (CLARKE *et al.*, 2000b). Nevertheless, two population genetic analyses based on SGE of five and 12 allozyme loci respectively, suggested that gene flow between *L. saxatilis* and *L. tenebrosa* is somehow reduced compared to gene flow within *L. saxatilis* (GOSLING *et al.*, 1998; WILSON *et al.*, 1999). However, there was no consistent pattern of differentiation between the taxa, and cluster analyses failed to separate *L. tenebrosa* from *L. saxatilis* as two homogeneous groups. Earlier allozyme work by JANSON & WARD (1985) also suggested that *L. tenebrosa* is a distinct form of *L. saxatilis* rather than a separate species. Similarly, allozyme analyses performed in the context of AMBIOS suggested that gene flow between *L. saxatilis* and *L. neglecta* may be reduced. However, once again there is no consistent pattern of differentiation or clustering as homogeneous groups. Moreover, RAPD analyses of *L. neglecta* were unable to separate this form from *L. saxatilis* (CROSSLAND *et al.*, 1996; WILDING *et al.*, 1998). In contrast, a survey of shell shape in six lagoonal and three coastal populations does clearly separate out *L. tenebrosa* from Golam Head, Ireland.

The situation for the H and M forms of *L. saxatilis* is complex. These animals occupy different shore levels, have different morphologies and show developmental differences (HULL *et al.*, 1996). At local scales, both forms can be differentiated by a few RAPD bands, even though this does not allow a consistent clustering (WILDING *et al.*, 1998). One 'diagnostic' RAPD band contained a microsatellite showing a differential size distribution between the two forms. This may suggest some degree of reproductive isolation. However, neither the restriction site analysis of the other 'diagnostic' RAPD band (X-80), nor the allozyme, calmodulin and mtDNA data allow a clear cut separation of these two forms. Similarly, the two forms show no consistent heat coma tolerance differences (CLARKE *et al.*, 2000b). On the other hand, there is good evidence that they mate assortatively by form (HULL, 1998; PICKLES & GRAHAME, 1999). It therefore seems that *L. saxatilis* H and M may be in the process of speciation without however having reached complete reproductive isolation and genetic differentiation.

In the course of the AMBIOS program still, another special ecotype of *L. saxatilis* was considered, viz. a 'barnacle dwelling' morphotype, referred to as B (CALEY *et al.*, 1995), differing from *L. neglecta* and *L. saxatilis*. Yet, neither the restriction fragment analysis of CAL-1, CAL-2, X-80 and DELETION, nor the RFLP and sequence analysis of Cyt*b* and COI, could consistently differentiate the B ecotype from *L. neglecta* or typical *L. saxatilis*, even though at some sites there was evidence for reduced gene flow between this latter and the B form. However, on the basis of allele frequencies, *L. saxatilis* B seems always to come out indistinguishable from *L. neglecta*, but the two morphs can be different from 'large' *L. saxatilis*. On the other hand, there is evidence that *L. neglecta* is an annual species, whereas *L. saxatilis* B is longer lived (L. JOHNSON, pers. comm.).

Azorean *L. saxatilis* (n = 54) was compared with *L. saxatilis* from Robin Hoods Bay (England), Vigo (Spain) and Venice (Italy) (n = 80), *L. arcana* from Robin Hoods Bay (England) (n = 20) and *L. striata* from the Azores (n = 20) by means of PAGE of eight polymorphic enzyme loci, 2DE of GP and IEF of EST. The allozyme data showed that the Azorean population was as variable as *L. saxatilis* from elsewhere and that its allele frequencies did not differ significantly from those of other *L. saxatilis* populations. These observations were fully supported by the EST and 2DE data. Hence it seems that, in terms of genetic variability and differentiation, Azorean *L. saxatilis* does not show the expected pattern of an introduced, peripheral and highly isolated population. This is somewhat surprising in a live-bearing species with limited (active) dispersal capacity. However, all allozyme studies performed in the context of AMBIOS did confirm the conspicuous shore level allele frequency differentiation at the AAT locus in *L. saxatilis* (WILSON & GOSLING, 1998) which has been attributed to strong selection (JOHANNESSON & JOHANNESSON, 1989; JOHANNESSON *et al.*, 1995).

Finally, the relationships of the three well-recognized rough periwinkles (*L. saxatilis* s.s., *L. arcana* and *L. compressa*) were investigated with allozymes, 2DE, nuclear restriction site polymorphisms, SSCP's of Cyt*b*, RFLP's of COI, and sequences of Cyt*b*, COI and nuclear rDNAs. The current allozyme and 2DE data suggest that *L. compressa* is the sister taxon of the *L. saxatilis* - *L. arcana* clade, and thus confirm most earlier allozyme studies based on SGE (e.g. WARD, 1990, KNIGHT & WARD, 1991). A similar result was obtained from the restriction site analysis of four nuclear genes (CAL-1, CAL-2, X-80 and DELETION), even though the topology of rough periwinkle relationships received little bootstrap support and was only evident when using NEI's (1978) genetic distances, but not when using WRIGHT's (1978) Prevosti distances. In contrast, the RFLP and sequence analysis of Cyt*b* and COI did not consistently separate the three rough periwinkles, made each of them paraphyletic and yielded mainly unresolved and poorly supported tree topologies. Furthermore, the SSCP analysis of another Cyt*b* fragment suggested a closer similarity between *L. arcana* and *L. compressa*, thus placing *L. saxatilis* outside this group. Although this result agrees with the allozyme data of BACKELJAU & WARMOES (1992), it should be stressed that the SSCP data were interpreted by ordination via non-metric multidimensional scaling, which is not a phylogeny inference technique allowing reconstruction of 'common descent'. Moreover, this approach does not provide support values, and hence the stability of this result cannot be evaluated. Finally, the 18S and 28S rDNA sequences yielded various, contradictory, but nevertheless bootstrap supported, topologies, depending on the taxa included and the gene fragments considered. Hence REID's (1996) morphology-based proposal to maintain an unresolved trichotomy for the rough periwinkles still seems to be the best reflection of the current knowledge on the relationships of these species.

Discussion

METHODOLOGICAL ISSUES

From a methodological point of view the AMBIOS program clearly demonstrated that not all techniques and molecular markers employed are equally suitable for measuring efficiently genetic diversity and inferring phylogenetic relationships. For example, despite its higher resolving power than conventional protein electrophoresis, 2DE was found to be a less efficient technique if large numbers of specimens are to be surveyed. Furthermore, the interpretation and statistical analysis of 2DE profiles in population genetic terms is still somewhat problematic.

Similarly, the genetic background of littorinid Mb variation still remains speculative (e.g. OLABARRIA *et al.*, 1998) and Mb therefore seems less appropriate for population genetic analysis. However, littorinid Mbs do appear useful for qualitative (eco)phenotypic analyses of variation, and for helping to answer taxonomic questions and problems of phylogenetic relationships (e.g. MEDEIROS *et al.*, 1998), particularly if analysed via IEF. This latter technique does indeed uncover cryptic variation that may remain undetected by PAGE or SGE (e.g. MEDEIROS *et al.*, 1998).

More surprising was the relative failure of microsatellites as a population genetic markers. The pervasive presence and high frequencies of tentative null alleles, the lack of cross-species amplification and the considerable laboratory efforts required to develop them (WINNEPENNINCKX & BACKELJAU, 1998b), mean that littorinid microsatellites appear to be less suitable for population genetic work than allozyme or RAPD data. This is not to say that, in general, the analysis of microsatellites is not worthwhile pursuing, but rather that, in this particular case, considerable extra effort would be needed to produce useful microsatellite data. Similarly, the calmodulin intron data do not seem to be very useful for population genetic work in *L. saxatilis*, but appeared to be informative for phylogenetic analyses of taxa above the species level.

In contrast, conventional allozyme electrophoresis (SGE and PAGE) and RAPD analyses appear to be highly cost-effective techniques for performing large scale surveys of genetic variation. However, the information yield per locus is higher with allozymes than with RAPD bands, because these latter behave as dominant/recessive markers so that heterozygote genotypes cannot be directly inferred from gel banding patterns (e.g. BOWDITCH *et al.*, 1993; LYNCH & MILLIGAN, 1994; BACKELJAU *et al.*, 1995).

Finally, the most efficient technique to screen genetic polymorphisms appears to be the combination of SSCP and DNA sequencing, even though the potential of this approach is still severely underscored. The virtues of SSCP reside in the relative simplicity of the experimental procedures, the possibility for inferring individual genotypes, the high resolving power that allows detection of point mutations, the possibility of screening large numbers of individuals, the use of known DNA fragments and the possibility of sequencing new allelic/haplotypic variants. Hence, SSCP analyses combine the advantages of allozymes, RAPDs, RFLPs, microsatellites and DNA sequencing!

CONCEPTUAL ISSUES

The investigations here presented illustrate convincingly that studying and conserving marine biodiversity requires a multidisciplinary approach that must be applied at different spatial scales. Both *L. striata* and *L. saxatilis* show considerable morphological and genetic variation, but the structuring and interpretation of the patterns of this variation may differ fundamentally. In *L. striata*, morphological differentiation is apparent at microgeographic scales and is maintained in the presence of intense gene flow, even over distances of up to 3000 km. In *L. saxatilis*, on the contrary, patterns of morphological variation may covary with different degrees of genetic exchange that are already noticeable at distances of a few meters (e.g. the H and M forms). These patterns of morphological variation and gene flow largely reflect the developmental differences between these periwinkles, and at the same time point to the possible role of selection and/or phenotypic plasticity in generating and maintaining marine biodiversity.

Generally, *L. striata* reveals a temporally stable, spatial structuring of shell variation, most probably in relation to wave-exposure and/or thermal stress, but shows little differentiation according to shore level or predation pressures, even though thermal stress and shore level effects may depend on the type of substrate (e.g. black vs light coloured rocks; cf. BRITTON, 1995). *L. saxatilis*, in contrast, shows strong effects of shore level and wave-exposure, but does not reveal a consistent pattern in thermal tolerance features. Given that these shore level differences may be accompanied by various degrees of molecular differentiation, it is obvious that classical taxonomic species boundaries only describe biodiversity at a relatively coarse level. Hence restricting the management of marine biodiversity to the species level will almost certainly result in important losses of evolutionary relevant variation present at the intraspecific level; this would have a serious and profound effect on evolutionary potential. Therefore it seems more appropriate to delimit 'Evolutionarily Significant Units' (ESU: historically isolated sets of populations that together encompass the evolutionary diversity of a taxon) and 'Management Units' (MU: sets of populations that are currently demographically independent) (MORITZ, 1994, 1996). In practice, these operational concepts are recognized on the basis of molecular markers such as mtDNA and allozymes, such that ESUs should be reciprocally monophyletic for mtDNA alleles and also differ significantly for allele frequencies at nuclear loci, whereas MUs only imply significant differences in allele frequencies, without considering a phylogenetic component. Hence an ESU is more inclusive than an MU.

Although the application of the ESU and MU concepts represents a fundamental recognition of the importance of intraspecific variation, and at the same time prevents man-

agement decisions being restricted to 'classically named' species, there are still a number of issues which should be considered if management policies were to be limited to the strict recognition of ESUs and MUs. The implementation of these concepts indeed relies solely on population inferences derived from 'neutral' molecular markers. This situation entails the danger that loci under selection may not receive the attention they merit as evolutionarily 'active' genetic components. In a similar way, the strict implementation of the ESU and MU concepts may not deal adequately with non-molecular diversity. This is well-illustrated in the present research program, where the observed morphological diversity could not be consistently interpreted in terms of ESUs or MUs. For example, the peculiar lagoonal form of *L. saxatilis*, viz. *L. tenebrosa*, shows a unique and consistent, apparently non-plastic, different shell morphology (e.g. JANSON & WARD, 1985), even though molecular techniques have so far failed to distinguish this form from other *L. saxatilis* (JANSON & WARD, 1985; GOSLING *et al.*, 1998; WILSON *et al.*, 1999). We therefore propose that the ESU and, even more so, the MU concepts be extended to also include significant differences for other than purely molecular traits. In this way, conservation managers may, for example, more appropriately consider ecologically relevant variation, which according to recent studies seems increasingly important in the formation of species. Indeed it is being argued that ecological separation may be as, or even more, important than geographical separation in the evolution of species (e.g. ORR & SMITH, 1998; MORELL, 1999). However, the logical and statistical framework for extending the ESU and MU concepts still needs to be established and entails a new difficulty, viz. whether or not, and how, to distinguish between genetic and plastic phenotypic variation. This issue will undoubtedly become more important as soon as attention is focused more on quantitative genetics, a topic which until recently has been neglected far too much in (marine) conservation biology (STORFER, 1996).

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