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# Microcell parasites of oysters: Recent insights and future trends

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**Abstract –** Our understanding of the microcell oyster parasites of the genera *Bonamia* and *Mikrocytos* has expanded in recent years with the application of ultrastructural and especially molecular biological research approaches. Molecular phylogenetic analyses of SSU rRNA genes have united three species, *Bonamia ostreae*, *Bonamia exitiosa*, and *Mikrocytos* (now *Bonamia*) *roughleyi*, in a microcell clade within the Haplosporidia, supporting both early and recent ultrastructural observations. Ultrastructural and molecular phylogenetic evidence has emerged that *Mikrocytos* mackini, on the other hand, is a unique protist with unusual adaptations for a parasitic existence. DNA probes and polymerase chain reaction (PCR) assays promise new insights into the life cycles, transmission, and diversity of these organisms. The development of *Ostrea edulis* lines selected for *B. ostreae* resistance will increase the viability of aquaculture industries for this species and, combined with rapidly developing biotechnological approaches for studying host defenses and host-parasite interactions, will allow greater insight into the nature of phenomena such as resistance and tolerance to disease in oysters.

**Key words:** Microcell / *Bonamia* / *Mikrocytos*

## 1 Introduction

Small, intracellular, protistan parasites caused serious mortality in the 1960s in Pacific oysters, *Crassostrea gigas*, at Denman Island, British Columbia, Canada (Bower 1988; Farley et al. 1988), and in flat oysters, *Ostrea edulis*, in California, USA (Katkansky et al. 1969). Parasites similar to the "microcells" observed by Katkansky et al. (1969) in California subsequently caused epizootic disease and mortality in *O. edulis* along the Atlantic coast of Europe (Comps et al. 1980; Van Banning 1986; Montes 1990; Hudson and Hill 1991; McArdle et al. 1991; Rogan et al. 1991) and in dredge oysters *Tiostrea lutaria* (syn. *chilensis*; Ó Foighil et al. 1999) in New Zealand, and were identified as the cause of winter mortality (Roughley 1926) in the Sydney rock oyster *Saccostrea commercialis* (syn. *glomerata*; Anderson and Adlard 1994) in southeastern Australia (Farley et al. 1988). They are now recognized as major parasitic threats to oyster populations worldwide (O.I.E. 2000).

Two genera and four species of microcells are currently acknowledged. *Bonamia* species include *Bonamia ostreae* (Pichot et al. 1980), believed to have been first observed by Katkansky et al. (Katkansky et al. 1969; Elston et al. 1986), which parasitizes *O. edulis* in Europe and in Washington, California, and Maine, USA (Barber and Davis 1994; Elston et al. 1986; Friedman and Perkins 1994; Friedman et al. 1989); and *Bonamia exitiosa* (Hine et al. 2001a), which infects *T. chilensis* in New Zealand (Dinamani et al. 1987). The genus *Mikrocytos* was proposed to include Denman Island disease agent *Mikrocytos mackini* and *Mikrocytos roughleyi*, the cause of winter mortality in *S. glomerata* (Farley et al. 1988). Similarity in appearance at the light microscope level (predominant cell forms are small (<5 $\mu$m) and roughly spherical, with relatively large, somewhat eccentric nuclei that give them a "fried egg" appearance (Bower et al. 1994) in histopathological sections), in cell specificity (most infect oyster hemocytes), and in transmission (all are directly transmitted among oyster hosts) led Farley et al. (1988) to conclude that these species were closely related. Indeed, we now know that *B. ostreae*, *B. exitiosa*, and *M. roughleyi* are (Carnegie et al. 2000; Cochennec-Laureau et al. 2003; Hine et al. 2001a), but *M. mackini* is not obviously related to members of any described taxon (Carnegie et al. 2003). The illumination of microcell interrelationships and phylogenetic affinities has broad implications, and with the development of molecular diagnostic tools for these protists is a significant recent development in microcell research. We review here the progress in microcell phylogenetics and molecular diagnostics, and revisit several aspects of microcell-oyster pathobiology. Finally, we review early progress in a major area of current and future research: the breeding for resistance to bonamiasis, and the use of bonamiasis as a model for illuminating cellular and molecular bases of host-parasite interactions.

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## 2 Phylogenetics of the microcells

The earliest ultrastructural study of *B. ostreae* revealed dense cytoplasmic structures resembling haplosporosomes (Pichot et al. 1980), features present in the Haplosporidia and Myxozoa (Perkins 1979) as well as the Paramyxea (Morris et al. 2000). The presence of these structures in *B. ostreae*, an organism not displaying the cell-within-a-cell structure of the Paramyxea, argued for inclusion in the Haplosporidia (Perkins 1987, 1988), a position further supported by the observation that *B. ostreae* at least occasionally displays multinucleate plasmodial forms (Brehélin et al. 1982). Because *B. ostreae* spores have never been observed, however, placement of this parasite in a phylum whose members are defined by their spores (Sprague 1979) is tenuous (Elston et al. 1986). Furthermore, *B. ostreae* was shown to pass directly between neighboring oysters (Elston et al. 1986; Poder et al. 1982). Direct transmission of *Haplosporidium* spp., except perhaps in the case of *Haplosporidium pickfordi* (Barrow 1965), has not been demonstrated.

Sequencing of the SSU rRNA gene of *B. ostreae* in the late 1990s made genetic analyses possible. The hybridization of putatively *B. ostreae*-specific polynucleotide (300 bp) *in situ* hybridization (ISH) probes to *Haplosporidium nelsoni* was a fortuitous result that provided the first molecular genetic evidence that *B. ostreae* may be a haplosporidian (Cochennec et al. 2000). Parsimony phylogenetic analyses using SSU rRNA genes then placed *B. ostreae*, with strong (100% bootstrap) support, in this phylum, with *H. nelsoni*, *Haplosporidium costale*, and *Minchinia teredinis* its closest relatives (Carnegie et al. 2000).

Like *B. ostreae*, both *B. exitiosa* and *M. roughleyi* parasitize host hemocytes and display haplosporosome-like structures and multinucleate plasmodial cell forms (Dinamani et al. 1987; Farley et al. 1988; Hine 1991, 1992; Hine and Wesney 1992, 1994; Hine et al. 2001a; Cochennec-Laureau et al. 2003). Molecular phylogenetic analyses confirmed their close relationship to *B. ostreae*. Hine et al. (2001a) found that the SSU rDNA of *B. exitiosa* and *B. ostreae* were 96.6% similar over 1623 bp. Cochennec-Laureau et al. (2003) then used parsimony analysis to place *B. exitiosa* and *M. roughleyi* with *B. ostreae* in the Haplosporidia. Parsimony analysis by these authors and Reece and Stokes (2003) supported the close relationship of these microcells to *H. costale* and *M. teredinis*, and Cochennec-Laureau et al. (2003) found furthermore that *B. exitiosa* and *M. roughleyi*, rather than *B. ostreae*, might be sister species (bootstrap support for this conclusion was a modest 69%). Phylogenetic analyses performed for this review support this relationship (Fig. 1); indeed, a single 10-nucleotide insertion/deletion notwithstanding, the SSU rDNA sequences of *B. exitiosa* and *M. roughleyi* are 99.5% similar over the 962 positions included in an alignment of the sequences deposited in the National (USA) Center for Biotechnology Information database (GenBank). SSU rDNA sequence data suggest therefore that the austral microcells, *B. exitiosa* and *M. roughleyi*, shared a common ancestor following divergence of their lineage from that of *B. ostreae*—and possibly following divergence of the microcells into northern and southern hemispheric forms.

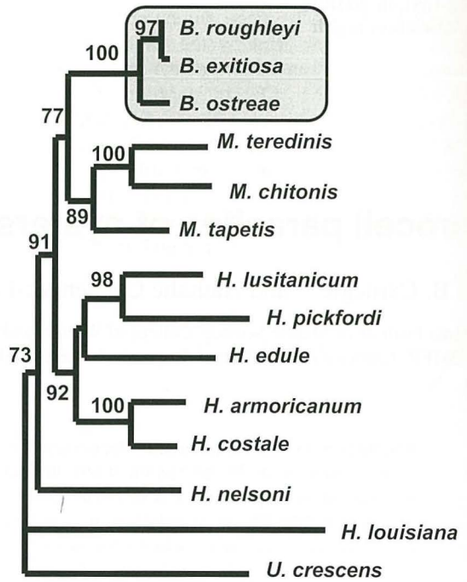


**Fig. 1.** Parsimony bootstrap tree of haplosporidian SSU rDNA indicating the position of *Bonamia* spp., including *B. roughleyi*, within the Haplosporidia. Numbers at nodes indicate percentage support of 1000 bootstrap replicates. The austral microcells *B. exitiosa* and *B. roughleyi*, based on available sequence data, appear to be sister species.

A close relationship of *M. mackini* to other microcells was dubious. While *Bonamia* spp. and *M. roughleyi* primarily parasitize oyster hemocytes (Balouet et al. 1983; Dinamani et al. 1987; Farley et al. 1988), *M. mackini* most obviously infects vesicular connective tissue cells (Farley et al. 1988), though also and heart and adductor muscle myocytes as well as hemocytes (Hervio et al. 1996; Hine et al. 2001). While advanced *Bonamia* spp. infections tend to become diffuse and systemic (Balouet et al. 1983; Dinamani et al. 1987; Hine 1991a), with infected hemocytes to be found in every host tissue (Elston et al. 1986), natural *M. mackini* infections always remain strongly focal (Farley et al. 1988; Hervio et al. 1996). *Mikrocytos mackini* has the widest experimental host range of the microcells, infecting not only *C. gigas* but *Crassostrea virginica*, *O. edulis*, and *Ostreola conchaphila* as well (Bower et al. 1997). Most significantly, *M. mackini* alone possesses neither haplosporosomes nor mitochondria (Hine et al. 1991), which led Hine et al. (2001) to conclude that *M. mackini* "is not a haplosporidian". Parsimony and evolutionary distance phylogenetic analyses supported this contention (Carnegie et al. 2003). These analyses also excluded *M. mackini*, however, from every other protistan phylum for which SSU rDNA sequences are available. The true phylogenetic position and affinities of *M. mackini* are unknown.

*Mikrocytos* was revealed to be a polyphyletic genus, and "microcell" a term with no phylogenetic significance. *Mikrocytos roughleyi* is, like *B. ostreae and B. exitiosa*, a microcell haplosporidian, and should be reassigned to the genus *Bonamia* (Cochennec-Laureau et al. 2003). This position is already embraced by the Office International des Epizooties, which lists *M. roughleyi* among the causative agents of bonamiasis (O.I.E. 2003). *Mikrocytos roughleyi* will be referred to as *Bonamia roughleyi* hereafter in this text.

The early review by Grizel et al. (1988) of research concerning *B. ostreae* and bonamiasis is still very relevant. Some aspects of the biology of the microcell haplosporidians and *M. mackini* and the diseases they cause are worth revisiting, however, in light of recent research and progress in microcell phylogenetics in particular.

### 2.1 Pathology and host range

Pathology and host range formed the initial basis for microcell taxonomy:

"*Mikrocytos* g. n. is always associated with focal abscesses and occurs in crassostreid oysters. *Bonamia* is always associated with generalized infections and only occurs in ostreid oysters (Farley et al. 1988)".

*Bonamia roughleyi* was originally assigned to the genus *Mikrocytos* even though, like *B. ostreae*, it infected the hemocytes of its oyster host (Farley et al. 1988). The phylogenetic affinity of *B. roughleyi* to the *Bonamia* spp. (Cochennec-Laureau et al. 2003), however, shows tissue tropism to be significant. *Microcell haplosporidians generally parasitize and proliferate in oyster hemocytes. Mikrocytos mackini* also occurs in hemocytes (as well as heart and adductor muscle myocytes; Hervio et al. 1996; Hine et al. 2001), but is more obviously associated with vesicular connective tissue cells (Farley et al. 1988) and does not appear to proliferate in hemocytes. The significance of a single record of *B. ostreae* infecting the gill epithelial cells of an unspecified number of very heavily infected oysters (Montes et al. 1994) is still unclear, though the gill epithelium may be portal of entry, as for *H. nelsoni* (Ford and Tripp 1996), or a point of egress from the host (Montes et al. 1994). More epitheliotropic microcell haplosporidians, on the other hand, may await discovery and description. Dr. P.M. Hine (pers. comm.) kindly suggests that, in histopathological sections of *Ostrea angasi* from southern Australia infected with a *Bonamia* sp., "gill epithelial cell infections are as common as hemocyte infections, and within the digestive gland, infection of the digestive diverticular epithelial cells is the rule, rather than the exception".

Advanced microcell haplosporidian infections indeed become systemic (Bower 2001; Cochennec-Laureau et al. 2003; Farley et al. 1988), but early or light infections are focal (Balouet et al. 1983; Bucke 1988; Bucke and Feist 1985; Dinamani et al. 1987; Elston et al. 1986; Friedman and Perkins 1994; Friedman et al. 1989; Hine 1991a; Zabaleta and Barber 1996). Gross surface lesions may be common (e.g., *B. roughleyi*; Farley et al. 1988), occasional (e.g., *B. ostreae*; Comps et al. 1980; Pichot et al. 1980), or rare (e.g., *B. exitiosa*; Dinamani et al. 1987).

*The host range of microcell haplosporidians is not limited to ostreid oysters.* In the early 1960s microcell epizootic in *O. edulis* in California (almost certainly caused by *B. ostreae*; Elston et al. 1986), *Crassostrea angulata* were also found to be infected (Katkansky et al. 1969); more recently, *Crassostrea rivularis* (syn. *ariakensis*) held in France were found to be infected by one *Bonamia* sp. (likely *ostreae*; Cochennec et al. 1998), and *C. ariakensis* in North Carolina, USA, with another, novel *Bonamia* sp. (Burreson et al. 2004). *Saccostrea glomerata*, the host for *B. roughleyi*, may be more closely related to *C. gigas* and *C. ariakensis* than to *Ostrea* spp. (Ó'Foighil et al. 1999). *Crassostrea gigas* is refractory to *B. ostreae* in the field (Balouet et al. 1983; Katkansky et al. 1969; Le Bec et al. 1991; Renault et al. 1994) or when challenged by injection in the laboratory (Culloty et al. 1999).

The natural host range for *B. ostreae*, currently *O. edulis* alone, is potentially broader, as *B. ostreae* is known to infect *C. angulata* (Katkansky et al. 1969), *T. chilensis* (Bucke and Hepper 1987; Grizel et al. 1983), *Ostrea denselamellosa* (Le Borgne and Le Pennec 1983), *O. angasi* (Bougrier et al. 1986), *Ostrea puelchana* (Pascual et al. 1991), and *C. ariakensis* (Cochennec et al. 1998). The documented host range of *B. exitiosa* is restricted to *T. chilensis* in New Zealand (Dinamani et al. 1987), and of *B. roughleyi* to *S. glomerata* in eastern Australia (Farley et al. 1988).

The potential host range for *M. mackini* is broad. In laboratory challenges (inoculation with a *M. mackini* cell suspension) and field trials at Denman Island, Bower et al. (1997) found that *O. edulis*, *O. conchaphila*, and *C. virginica* were perhaps more susceptible to *M. mackini* than *C. gigas* was. They also found that in *C. gigas*, *C. virginica*, and *O. edulis* infection after inoculation with a parasite suspension developed at 9.2 °C but not at 17.9 °C. Considering the host species above and their relatives, *M. mackini* is a potential threat to a wide range of oyster species in cooler waters worldwide.

### 2.2 Cell forms and life cycles

Predominance of a small (<5 $\mu$m), uninucleate cell form is characteristic of microcell haplosporidians (Pichot et al. 1980; Dinamani et al. 1987; Cochennec-Laureau et al. 2003). These cells may be more electron-dense (ribosome-rich) or electron-clear (Pichot et al. 1980; Dinamani et al. 1987; Hine 1991a,b), or, in the case of *B. exitiosa*, may or may not contain a large vacuolar mitochondrion among their mitochondria (Hine et al. 2001a). Individual cells may be intermediate in appearance (Dinamani et al. 1987; Hine et al. 2001a), suggesting that "dense" and "clear" may represent end points on continua. The significance of these states is still not clear. The dense form of *B. ostreae* is more numerous in heavily infected oysters, and the clear form is more common in light infections. The dense form is presumed to be the infective stage (Pichot et al. 1980). In *B. exitiosa*, Hine (1991) observed seasonal changes in the frequencies of dense and clear forms, with infective dense forms occurring in more oysters in every month except August and September, when clear forms occurred more frequently.

*Multinucleate plasmodial forms are expressed by all known microcell haplosporidians but make a questionable contribution to disease transmission.* Only *B. exitiosa* plasmodia occur regularly (Hine 1991; Hine et al. 2001a). *Bonamia ostreae* plasmodia have been observed very rarely and only in moribund or post-mortem oysters (Brehélin et al. 1982); *B. roughleyi* plasmodia are rare as well

(Cochennec-Laureau et al. 2003). The hypothesis that development of multiple infective cells from multinucleate plasmodia in even relatively few dead oysters is a means by which *B. ostreae* proliferates explosively in an oyster population (Brehélin et al. 1982) remains to be tested. Infrequently occurring plasmodia may simply be a vestige of an ancestral haplosporidian sporogenic pathway (Hine 1991) obviated in microcell haplosporidians with the evolution of directly infective dense forms.

Few generalizations can be made regarding seasonal infection cycles of microcell haplosporidians. *Bonamia exitiosa* infections display a strong seasonality tied closely to *T. chilensis* gametogenesis and reproduction (Hine 1991a,b). Infection intensity peaks from January to April, parasites proliferating when hemocytes migrate into the gonad to resorb unspawned gametes (Dinamani et al. 1987; Hine 1991a,b). Mortality and transmission of *B. exitiosa* occur primarily from January-August, in the months following proliferation in the gonad (Hine 1991a). Accumulating evidence for *B. ostreae*, on the other hand, offers no support for a close association with gonad (contrary to Van Banning 1990) or for strong seasonality of infections. Prevalence and intensity in general are greatest in warmer months, but infections and mortality occur year-round (Balouet et al. 1983; Tigé and Grizel 1984; Montes and Melendez 1987; Montes et al. 1994; Culloty and Mulcahy 1996) and are unrelated to gonadal development (Cáceres-Martínez et al. 1995). The seasonality of *M. roughleyi* infections is uncharacterized, but gross signs of infection and winter mortality occur primarily in August and September (Adlard and Lester 1995).

In *M. mackini*, Hine et al. (2001) identified three distinct cell types based on ultrastructural characteristics and tissue tropism, and proposed a developmental cycle in which the parasite is transformed from one cell type to another while cycling between vesicular connective tissue and myocytes via hemocytes. Their most striking observation was the frequent close association of *M. mackini* with host cell nuclei (*M. mackini* sometimes occurred *within* host cell nuclear membranes) and mitochondria (*M. mackini* itself is amitochondriate). A physical affinity for host cell mitochondria in particular could only have evolved after these organelles had already been acquired by eukaryotes. Conceivably, this could be the first evidence that *M. mackini*, like the Microspora, is a derived organism that has lost its mitochondria in adapting to a parasitic existence. Alternatively, *M. mackini* may be an ancient, amitochriate protist that has more recently evolved a facultative parasitic relationship with oyster hosts.

The annual life cycle of *M. mackini* is still unknown. However, while *M. mackini* is typically detectable histopathologically only between March and June, polymerase chain reaction (PCR) experiments have shown it to be associated with oysters year-round (Carnegie et al. 2002).

### 2.3 Undescribed microcells

Microcell haplosporidians in *T. chilensis* in Chile (Campalans et al. 2000), *O. angasi* in southern and western Australia (Hine and Jones 1994), and *C. ariakensis* in North Carolina, USA (Burreson et al. 2004) await definitive identification. Microcells observed in *C. gigas* in Hawaii and *Ostrea lurida* (= *O. conchaphila*) in Oregon (Farley et al. 1988) may have been *M. mackini* (or a relative), as vesicular connective tissue cells were infected.

## 3 Molecular diagnostics

The development of microcell-specific molecular diagnostic tools was imperative because the small size of these parasites makes them difficult to detect using standard histopathology and histocytology. Immunoassays for *B. ostreae* (Boulo et al. 1989; Mialhe et al. 1988; Cochennec et al. 1992) held great early promise. The monoclonal antibodies designed for *B. ostreae* from France (Mialhe et al. 1988) reacted only weakly with *B. ostreae* from Maine (Zabaleta and Barber 1996), however, suggesting that serological differences between *B. ostreae* strains could limit the usefulness of antibody-based techniques. The first DNA-based diagnostic assay for a microcell was designed for *B. roughleyi*. Adlard and Lester (1995) used universal primers to amplify the internal transcribed spacer (ITS) region between the SSU rRNA and LSU rRNA genes, producing amplicons that differed in size between *B. roughleyi* (680 bp) and *S. glomerata* (1500 bp). Questions of specificity and sensitivity, however, limit the usefulness of this PCR. Because the primers were not specific, they might amplify the rDNA not only of *B. roughleyi* or *S. glomerata* but of contaminant organisms too. The 680-bp product characteristic of *B. roughleyi* could also be characteristic of other protists, or contaminating bacteria or fungi. The sensitivity of the assay relates to its specificity. Both forward primer (a perfect match to the target sequence in the congeneric oyster species *Saccostrea cucullata*, GenBank acc. no. AJ389634; the *S. glomerata* sequence is unknown) and reverse primer (87.5% identical to the target sequence in *S. glomerata*, acc. no. Z29552) should hybridize to oyster DNA template in the PCR reaction. Even if not producing amplification, such interactions can reduce the efficiency of parasite-specific PCR. Adlard and Lester (1995) did, however, detect one *B. roughleyi* cell among about 400 host cells.

Cochennec et al. (2000) and Carnegie et al. (2000) presented as "*B. ostreae*-specific" PCR assays that are now known to be broader in specificity. Based on target DNA sequence similarity, the Cochennec et al. (2000) assay should detect the SSU rDNA of all microcell haplosporidians. The Carnegie et al. (2000) assay should detect *B. ostreae* and *B. exitiosa* but perhaps not *B. roughleyi*, which has a three-nucleotide deletion in the target site for the forward primer (the *B. roughleyi* sequence at the reverse primer binding site is not known). Neither assay amplifies oyster DNA. Both assays have undergone some validation against histocytological diagnosis, the light microscopic screening of fixed and stained hemolymph smears ($n$ = 185 (Carnegie et al. 2000); $n$ = 28 (Diggles et al. 2003). (Histocytology is no less sensitive than histopathology for detecting *B. ostreae* (Culloty and Mulcahy 1996; Zabaleta and Barber 1996) and perhaps more sensitive for detecting *B. exitiosa* (Diggles et al. 2003), but more rapid and less expensive.) Assuming that the histocytologically determined infection intensity scores between species are roughly



equivalent, the two assays might reasonably be compared. Both assays detected every "heavy" and "moderate" infection (Carnegie et al. 2000: 8/8 and 16/16, respectively; Diggles et al. 2003: 2/2 and 4/4). Both detected most "light" and "scarce" infections (Carnegie et al. 2000: 13/15 and 20/30; Diggles et al. 2003: 6/6 and 2/4). Dividing the number of infections detected by PCR alone by the number detected by histocytology alone generates an index of sensitivity. The Carnegie et al. (2000) assay detected 3.7 times more *B. ostreae* infections than histocytology. The assay validated by Diggles et al. (2003) detected 4.0 times more *B. exitiosa* infections than histocytology. While these assays still need to be validated directly against one another, they appear to be roughly equivalent in sensitivity.

PCR-restriction fragment length polymorphism (PCR-RFLP) assays introduced to distinguish *B. ostreae* from *B. exitiosa* (Hine et al. 2001) and to distinguish *B. roughleyi* from the other *Bonamia* spp. (Cochennec-Laureau et al. 2003) may provide the most straightforward molecular means for distinguishing among microcell haplosporidians. Both assays rely upon the PCR described by Cochennec et al. (2000) for amplification of *B. ostreae*. The PCR reaction mixture should include PCR buffer at 1X concentration, 2.5 mM $MgCl_2$, 0.2 mM dNTP's, 1.0 $\mu$M primers (forward, BO: CATTTAATTGGTCGGGCCGC; reverse, BOAS: CTGATCGTCTTCGATCCCCC), 0.02 units $\mu l^{-1}$ *Taq* DNA polymerase, and 0.2 ng $\mu l^{-1}$ template DNA. The reaction should begin with template DNA denaturation for 5 min at 94 °C, followed by 30 cycles of 94 °C for 1 min/55 °C for 1 min/72 °C for 1 min, and followed finally by a final extension for 10 min at 72 °C. A 300-bp *B. ostreae* product would be digested by both *Hae* II (producing fragments of 115 and 189 bp) and *Bgl* I (120 and 180 bp). A 304-bp *B. exitiosa* product would be digested by *Hae* II (producing 115- and 189-bp fragments) but not by *Bgl* I. A 304-bp *B. roughleyi* PCR product would not be digested by either enzyme.

Specific *in situ* hybridization (ISH) assays for the microcell haplosporidians await development. Cochennec et al. (2000) detected *B. ostreae in situ* with a digoxigenin-labeled, polynucleotide (300 bp) probe. However, *H. nelsoni* was detected too, indicating that this assay might be phylum-specific. The cocktail of three fluorescein-labeled oligonucleotide probes that Carnegie et al. (2003a) used to detect *B. ostreae in situ* (Fig. 2a) did not hybridize to *H. nelsoni*, suggesting that this assay was more specific. However, the target sites for at least two of the oligonucleotides used by Carnegie et al. (2003a) were strongly conserved in at least one other microcell (*B. exitiosa*), suggesting that this ISH assay may not be more than microcell haplosporidian-specific. With at least partial sequences available now in GenBank for *B. ostreae*, *B. exitiosa*, and *B. roughleyi*, the probes for the Carnegie et al. (2003a) assay can be redesigned for species specificity. In the meantime, the assay as currently designed is most useful for distinguishing microcell haplosporidians as a group from *M. mackini*.

PCR and fluorescent ISH assays for *M. mackini* (Fig. 2b) were also recently developed (Carnegie et al. 2003). The prevalence of *M. mackini* in 1056 *C. gigas* was estimated to be 3.5 times higher by PCR than by microscopic methods, including standard histopathology plus stained imprints of as many as three gross pustules per individual; 15 times as many oysters were *M. mackini*-positive by PCR alone as by microscopy alone. SSU rDNA from *Bonamia ostreae* was not amplified.

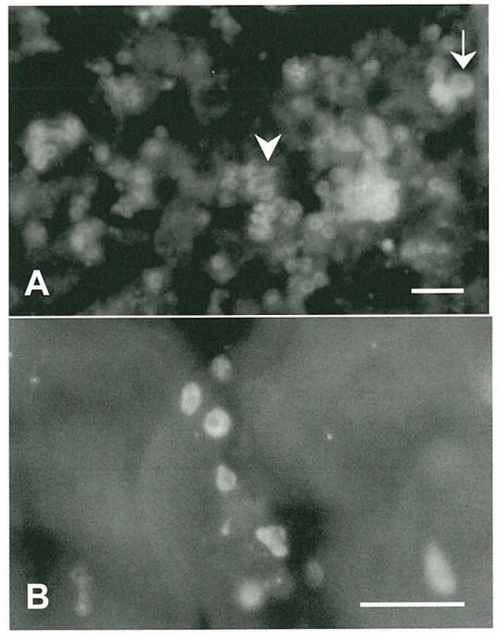


**Fig. 2.** Detection of microcells by fluorescent *in situ* hybridization. 2A. Numerous *Bonamia ostreae* in hemocytes infiltrating *Ostrea edulis* gill tissue. A larger *B. ostreae* is indicated by an arrow, and a cluster of smaller cells by an arrowhead. Scale bar = 10 $\mu$m. 2B. *Mikrocytos mackini* cells in the adductor muscle of experimentally infected *O. edulis*. Scale bar = 10 $\mu$m.

## 4 Future trends

Selective breeding for resistance to bonamiasis and the use of bonamiasis as a model for illuminating cellular and molecular bases of host-parasite interactions are major areas of current work that hold great promise for the future. These lines of research are also closely related. The use of crosses between lines selectively bred for a performance trait such as resistance to bonamiasis can be useful in mapping quantitative trait loci (QTLs) and ultimately identifying the genes underlying this trait – providing a window into the molecular basis of a host-parasite interaction. Genes found responsible for resistance to bonamiasis may then be used as biomarkers – in their allelic states or expression levels – for resistant stocks. Genes or proteins involved in host-parasite interactions may also be used as biomarkers for the physiological (or infection) state of an individual animal. The following is a synopsis of recent progress in these areas.

### 4.1 Selective breeding for disease-resistant or -tolerant oysters

Genetic improvement has been achieved with many significant oyster diseases through selection of individuals that appear more resistant to the disease as broodstock. Examples have been reported, for example, for *H. nelsoni* (Haskin and Ford 1978; Haskin and Ford 1979; Paynter et al. 1997) and Juvenile Oyster Disease (JOD) (Barber et al. 2000; Davis et al. 1997; Farley et al. 1997) in *C. virginica*.

By the mid-1980s there was strong evidence that *O. edulis* might be capable of developing resistance to *B. ostreae*. Elston et al. (1987) showed that members of a "carrier" *O. edulis* population (30% *B. ostreae* prevalence) descended from the original *B. ostreae*-challenged *O. edulis* population from California (Katkansky et al. 1969) exhibited dramatically better survival (74%) than members of a presumably naïve population from Maine (1%). Since 1985, scientists at IFREMER (France) have selectively bred *O. edulis* for resistance to *B. ostreae*. Contrary to the programs developed for resistance to *H. nelsoni* or *B. roughleyi*, experimental infections were used to increase and control the pressures of selection (Cochennec 2001; Culloty and Mulcahy 1992; Hervio 1992; Mialhe et al. 1988). Mass selection allowed sufficient numbers of oysters to survive to permit selection and comparison with natural oyster survival levels (Baud et al. 1997; Martin et al. 1993; Naciri 1994; Naciri-Graven et al. 1999). In 1992, the program was reorganized to evaluate the heritability of resistance and to reduce the risk of inbreeding (Naciri-Graven et al. 1998). Third generation selected oysters showed significantly higher survival rates than non-selected oysters (52.3% versus 2.5%). Moreover, the survival rates were correlated with *B. ostreae* prevalence (Bédier et al. 2001). Resistance to *B. ostreae* infections is clearly heritable, and mainly (but not entirely) additive (Naciri-Graven et al. 1998). The nature of resistance to bonamiasis appears to be increased tolerance of *B. ostreae* infections (Naciri-Graven et al. 1998); management of infections such that they remain light and, to a point, have a minimal impact on growth and reproductive output.

The French *O. edulis* selection program has focused on improving growth performance as well as disease resistance. The economic impact of the disease can be reduced by improving survival during the second and third year of growth, as well as by obtaining oysters of commercial size more quickly.

Selection for bonamiasis-resistant oysters has also been achieved in Ireland (Hugh Jones 1999; Culloty et al. 2001). In Cork Harbour, oysters alive after four years of *B. ostreae* exposure have been used as broodstock. The progeny of these oysters developed fewer *B. ostreae* infections of lower intensity relative to naïve oysters of a similar age, and exhibited reduced mortality in Cork Harbour. Culloty et al. (2001) found no relationship between *B. ostreae* resistance and growth rate. (Naciri-Graven et al. 1999, on the other hand, found in France that oysters selected for resistance did tend to grow faster.)

In the Sydney rock oyster *S. glomerata*, no progress has been made as yet in breeding for resistance to *B. roughleyi* (Nell et al. 2000). Triploid *S. glomerata*, however, show reduced winter mortality relative to diploid animals (Hand et al. 1998).

### 4.2 Bonamiasis as an experimental model for host/pathogen interactions

The microsatellite markers developed in French laboratories (Naciri-Graven et al. 2000; Launey et al. 2001) should find a usefulness beyond the marker-assisted selection programs in which they are currently employed. Microsatellites and then QTLs may be mapped onto *O. edulis* chromosomes, and genes subsequently identified that underlie the tolerance (or susceptibility) to bonamiasis. Already, however, bonamiasis has emerged as a model for study of molluscan intracellular parasitism that is particularly interesting because of the double role played by the hemocytes in pathogenesis. Hemocytes function in non-specific defense at the same time that they are infected by *B. ostreae*.

Development of purification techniques for isolating *B. ostreae* (Mialhe et al. 1988) and of primary, short term hemocyte cell culture methods (Mourton et al. 1992) have facilitated *in vitro* analyses of the early stages of parasite detection and host cell penetration and of the phagocytic mechanisms applied to destroy the parasites. Hemocytes of both *O. edulis* and *C. gigas* phagocytosed *B. ostreae*, but *C. gigas* hemocytes were capable of destroying the parasite. Cytochalasin B reduced penetration by the parasite, suggesting that penetration is indeed active phagocytosis directed by the host (Chagot et al. 1992). Additional active involvement by the parasites in penetrating the host hemocytes was investigated using flow cytometry. Live and heat-killed parasites confirmed the existence of such a mechanism, as live parasites were internalised at a greater rate than heat-killed parasites. Internalisation of *B. ostreae* thus uses two pathways, one directed by host phagocytosis and the other directed by the parasite itself (Cochennec 2001).

Mixing sugars (mannose, glucose, fucose, N-acetyl glucosamine and galactosamine) with hemocytes does not affect phagocytosis of *B. ostreae* by oyster hemocytes, so lectin receptors on the surface of the hemocytes play a questionable role in *B. ostreae* internalisation (Chagot et al. 1992). Incubation of *B. ostreae* with similar sugars, before exposure to oyster hemocytes, decreased infection rates of both large and small agranular cells of *C. gigas,* and granulocytes and small agranular cells of *O. edulis*, suggesting that lectin-like receptors may be operative on the surface of the parasites (Chagot 1989). Using flow cytometry it was possible to discriminate granular from agranular cells on the basis of hemocyte lectin distributions (Auffret, pers.comm.). Granular cells had mannose, glucose, galactose, fucose, N-acetyl glucosamine residues on their cytoplasmic membranes, while agranular cells had mannose residues only. The presence of similar lectins on the surface of *B. ostreae* suggests that these lectins may play a significant role in hemocyte-parasite recognition and internalisation. It is possible that the parasite may mimic granular cell membranes in order to evade phagocytosis by granular hemocytes (Cochennec 2001).

Internalisation of *B. ostreae* by hemocytes of both *O. edulis* and *C. gigas* suggests that the barrier of specificity is not located only at the level of the hemocyte membrane. Hemocytes of *C. gigas* appear capable of eliminating the parasite. Chagot (1989) found no difference between the respiratory burst activities, measured by chemiluminescence, of the hemocytes from each species of oyster.



Additional investigations on lysosomal activities and enzymatic cellular characterisation using API-ZYM galleries, however, revealed significant differences between the hemocytes of the two oyster species (Xue 1998). Enzymatic activities were higher in the hemolymph of *C. gigas* than in the hemolymph of *O. edulis*. Moreover, the distribution of the activities among the total hemolymph, cell-free plasma, and the hemocytes was different between both oyster species. Enzyme activities were higher in the total hemolymph and the plasma fraction of *C. gigas*, while higher in the total hemolymph and hemocytes in *O. edulis*. Six post-phagocytic enzymatic activities (esterase, aminopeptidase, galactosidase, cathepsines, myeloperoxydase and production of $H_2O_2$) were evaluated for each hemocyte fraction. All activities were higher in granular cells than in agranular, large and small cells, confirming significant role of granular cells in the degradation of particles after phagocytosis (Cochennec 2001).

Irish research has focused recently on enzymatic and protein concentration differences between *B. ostreae*-infected and -uninfected *O. edulis*. Cronin et al. (2001) found hemolymph protein levels to be highly variable between season, site, and oyster strain, no correlation between infection level and lysozyme concentration, and no difference in total hemolymph protein levels between infected and uninfected oysters (though highly infected oysters had slightly depressed protein levels). Culloty et al. (2002) found no differences in amylase or aspartate aminotransferase between infected and uninfected oysters.

Resistant oyster strains offer new opportunities for the study of cellular defense mechanisms. Analyses of the distribution of hemocyte types showed that the large agranular cells were significantly less abundant, and that granulocytes were significantly more abundant, in oysters selected for their resistance compared with control susceptible oysters. Although it is not yet clearly established that the reduction in the proportion of large agranular cells is the consequence, or the cause, of proliferation of the parasite, these results show the importance of the proportion of each hemocyte type and their potential respective roles in the susceptibility of oysters *B. ostreae* (Cochennec 1997, 2001). The search for possible functional differences between hemocyte types showed that the large agranular cells had lower enzymatic properties than granular cells (Cochennec 1997, 2001; Cochennec-Laureau et al. 2003). In order to define the possible relationship between enzymatic parameters and resistance to bonamiasis, various susceptible and resistant oyster populations were compared. A hypothesis for the development of bonamiasis was proposed (Cochennec 2001): *B. ostreae* is internalised by the three hemocyte types. Phagocytosis is directed by the hemocytes, but *B. ostreae* takes an active part in its internalisation. Similarity between membrane lectins suggests that *B. ostreae* actively targets agranular cells which present relatively lower lytic enzymatic activity. Moreover, *B. ostreae* seems to influence the distribution of hemocyte types, in particular reducing the number of circulating granulocytes (Cochennec-Laureau et al. 2003); this phenomenon requires further clarification. Once internalised, *B. ostreae* is "hidden" within the large agranular cells and can survive the relatively lower enzymatic activity levels. In addition, *B. ostreae* appears capable of inhibiting the post-phagocytic respiratory burst, further enhancing survival (Hine and Wesney 1994).

In oysters selected for apparent resistance to bonamiasis, survival of the parasite seems to be compromised by the more active non-specific esterase activities of the large agranular cells, compared with that of the granulocytes. This esterase activity does not seem to be specific to infection by the parasite *B. ostreae*. These cytological parameters are quantifiable, and thus may be markers useful for selection programs aimed at selection for tolerance to bonamiasis.

## 5 Conclusion

The combination of selective bred, *B. ostreae*-resistant *O. edulis* lines and an expanding arsenal of molecular tools promises significant advances in our understanding of bonamiasis. Not insignificantly, this may expand our understanding of enigmatic haplosporidians such as *H. nelsoni* too. While significant differences may exist between the as yet unresolved but likely indirect life cycles of familiar haplosporidians such as *H. nelsoni* and the directly transmission of microcell haplosporidians, large components of the biochemistries of these organisms must surely be conserved. Discoveries concerning *B. ostreae*, therefore, may be relevant not only to *B. exitiosa* and *B. roughleyi* but to other haplosporidians as well.

*Mikrocytos mackini* is a unique organism with unusual adaptations for a parasitic existence. Its true phylogenetic affinities and distribution should soon become more clear.

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