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Microcell parasites of oysters: Recent insights and future trends

Ryan B. Carnegie1,a and Nathalie Cochennec-Laureau2

Abstract – Our understanding of the microcell oyster parasites of the genera Bonamia and Mikrocystos 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 Mikrocystos (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 Mikrocystos 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 / Mikrocystos

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 Tiostra lutaria (syn. chilensis; ÓFoighil 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 Mikrocystos was proposed to include Denman Island disease agent Mikrocystos mackini and Mikrocystos 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 µm) 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.
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 Haplosporida and Myxozoa (Perkins 1987), 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, argues for inclusion in the Haplosporida (Perkins 1987, 1988), a position further supported by the observation that B. ostreae at least occasionally displays multineucleate plasmodial forms (Brehelin et al. 1982). Because B. ostreae sporules have never been observed, however, placement of this parasite into 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 rDNA 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 (Dinamani et al. 1987) was a breakthrough for the first molecular genetic evidence that B. ostreae may be a haplosporidian (Cochenne-Laurée et al. 2000). Parsimony phylogenetic analyses using SSU rDNA genes then placed B. ostreae, with strong (100% bootstrap) support, in this phylum, with H. nelsoni, Haplosporidium costale, and Micronia terndenis its closest relatives (Carnegie et al. 2000).

Like B. ostreae, B. exitiosa and M. roughley parasitize host hemocytes and display haplosporosome-like structures and multineucleate plasmodial cell forms (Dinamani et al. 1987; Farley et al. 1988; Hine 1991, 1992; Hine and Wesney 1992, 1994; Hine et al. 2001a; Cochenne-Laurée 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 1187 bp. Cochenne-Laurée et al. (2003) then used parsimony analysis to place B. exitiosa and B. roughley with B. ostreae in the Haplosporida. Parsimony analysis by these authors and Recce and Stokes (2003) supported the close relationship of these microcells to H. costale and M. terndenis, and Cochenne-Laurée et al. (2003) found furthermore that B. exitiosa and M. roughley, 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 104-bp nucleotide insertion/deletion notwithstanding, the SSU rDNA data of B. exitiosa and B. roughley 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 astral microcells, B. exitiosa and M. roughley, shared a common ancestor following divergence of their lineage from that of B. ostreae. This divergence event occurred between the microcells into northern and southern hemispheric forms.

A close relationship of M. mackini to other microcells was dubious. While Bonamia spp. and B. roughley primarily parasitize oyster hemocytes (Balout 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 (Hine et al. 1996; Hine et al. 2001). While advanced Bonamia spp. infections tend to become diffuse and systemic (Balout et al. 1983; Dinamani et al. 1987; Hine 1991a), with infected hemocytes to be found in every host tissue (Elsweeney et al. 1986), natural M. mackini infections always remain strongly focal (Farley et al. 1988; Hervio et al. 1996). Microcytosis mackini has the widest host range of the microcells, infecting not only C. gigas but Crassostrea gigas, O. edulis, and Ostrea edulis conchaphila as well as Bower et al. (1986), that of M. mackini alone possesses neither haplosporosomes nor mitochondria (Hine et al. 1991), which led Hine et al. (2001) to conclude that mackini “is not included in the Haplosporida, in part as an outgroup for the putative mycelial/microcell clade” (e.g., E. B. Roughley; 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. 2003). The host range of microcellular haplosporidians is not limited to oyster hosts. In the early 1960s microcellular epizootics in O. edulis in California (almost certainly caused by B. ostreae; Bower et al. 1986) and Crassostrea angarctica were also found to be infected (Katkanisky et al. 1969); more recently, Crassostrea rivullos (syn. ariakensis) held in France were found to be infected by one Bonamia sp. (likely ostreae; Cochenne et al. 1998), and C. ariakensis in North Carolina, USA, with another, novel Bonamia sp. (Burreson et al. 2004). Successor epizootics, the host for roughley, may be more closely related to C. gigas and C. ariakensis than to Ostrea spp. (O’Tohill et al. 1990). Crassostrea gigas is refractory to B. ostreae in the field (Balout et al. 1983; Katkanisky et al. 1969; Le Benc et al. 1991; Renaut et al. 1994) or when challenged by injection in the laboratory (Culloty et al. 1999).

The potential host range for B. ostreae, currently O. edulis alone, is potentially broad, as B. ostreae is known to infect C. angarctica (Katkanisky et al. 1969), T. chilensis (Buck and Hepper 1987; Grizelle et al. 1983; Ostrea denselamellosa (Le Bonge and Le Pencine 1983), O. angasi (Bougrier et al. 1991), and Ostrea puebla (Carnegie et al. 1998), and C. ariakensis (Cochenne-Laurée 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. roughley 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. They also found that in C. gigas, C. virginica, and O. edulis infection af- ter inoculation of parasites with a paraotic suspension developing 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 μm), unicellular cell form is characteristic of microcellular haplosporidians (Pichot et al. 1980; Dinamani et al. 1987; Cochenne-Laurée et al. 2003). These cells may be more electron-dense (ribosome-rich) or electron-clear (Pichot et al. 2000; Dinamani et al. 1987; Hine 1991a,b), perhaps due to the presence of a large nucleus. As a result of these characteristics, the microcellular 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 microcellular forms. Microcellular forms contribute to disease transmission. Only B. exitiosa plasmodia occur regularly (Hine 1991; Hine et al. 2001a). Bonamia ostreae plasmodia have been observed very rarely (Amin et al. 1987; Hine et al. 2001a), suggesting that “dense” and “clear” may represent end points on a continuum. 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 frequency 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.
3 Molecular diagnostics

The development of microcell-specific molecular diagnostic tools was imperative because the small size of these parasites makes it difficult to detect using standard histopathology. Infection intensity peaks from January to April, parasites proliferating when hemocytes migrate into the gonad to resorb unspawned gametes (Dinamani et al. 1987; 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-Laureau et al. (2000) for amplification of B. ostreae. The PCR reaction mixture should include PCR buffer at IX concentration, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 1.0 uM primers (forward, 5'GGGTTAGTACAAGGCTAAGGCGG-3', reverse, 5'GGTCTTTTTTGTTTTGTTTGAAGGC-3'), 0.02 units/µl Taq DNA polymerase, and 0.2 ng/µl target template DNA. The reverse primer is complementary to a 69-bp target product characteristic of other protists, or contaminating bacteria or fungi.

Specific in situ hybridization (ISH) assays for the microcell haplosporidians await development. Cochennec-Laureau et al. (2000) detected B. ostreae in situ with a digoxigenin-labeled, polyamido- nitrile (300 bp) probe. However, neither monochloroacetic acid (McA) nor ethidium bromide (EB) would digest the bacterial rDNA band of 189 bp and 180 bp. A 304-bp B. ostreae product would be digested by dig (producing 115- and 189-bp fragments) but not by EB. A 304-bp B. roughleyi PCR product would not be digested by either enzyme.

Future trends

Selective breeding for resistance to bonamiosis and the use of bonamiosis 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 bonamiosis can be used in mapping quantitative trait loci (QTLs) and ultimately identifying the genes underlying this trait—a powerful window into the molecular basis of a host-parasite interaction. Genes found responsible for resistance to bonamiosis 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 published for the works of Haskin and Ford 1978; Haskin and Ford 1978; 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 Katanosky 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. ostreae or C. virginica, the programs were focused to increase and control the pressures of selection (Cocheenne 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 explicitly increase the heritability of resistance and to reduce the risk of inbreeding (Bédié et al. 1998). Third generation selected oysters showed significantly higher survival rates than non-selected oysters (52.5% versus 2.5%). Moreover, the survival rate of B. ostreae-resistant oysters increased to 61% in 1997. Oysters selected for B. ostreae resistance showed significantly higher survival rates than non-selected oysters (52.5% versus 2.5%).

Moreover, the survival rate of B. ostreae-resistant oysters increased to 61% in 1997. Oysters selected for B. ostreae resistance showed significantly higher survival rates than non-selected oysters (52.5% versus 2.5%).

5 Conclusion

The combination of selective breed, B. ostreae-resistant O. edulis lines and an expanding arsenal of molecular tools promises significant advances in our understanding of oyster disease. Not insignificant, this may expand our understanding of pathogenic and non-pathogenic oyster fishes. Pathological affinities and distribution should soon become more clear.

Acknowledgements. N. Cocheenne-Laureau’s contribution has been funded by European Community (Program FAIR DISEV CP78-4129). We thank Sharon McGladden for assistance with the manuscript.

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