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DIFFERENTIAL DIAGNOSIS OF MIXED *HAPLOSPORIDIUM COSTALE* AND *HAPLOSPORIDIUM NELSONI* INFECTIONS IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, USING DNA PROBES

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ABSTRACT *Haplosporidium costale* and *Haplosporidium nelsoni* are morphologically similar pathogens of the eastern oyster *Crassostrea virginica*. In the absence of the spore stage, infections of the two species are extremely difficult, if not impossible, to distinguish using traditional light microscopy of stained tissue sections. Species-specific molecular diagnostics were developed for *H. costale* from the small subunit ribosomal DNA (SSU rDNA) sequence. The polymerase chain reaction (PCR) primers amplified a 557 base pair (bp) region of the *H. costale* SSU rDNA, but did not amplify DNA from oyster (*C. virginica*) or from six other haplosporidans (*H. nelsoni, H. louisiana, H. lusitanicum, Minchinia teredinis, M. chitonis*, or *M. tapetis*). The DNA probe was used with *in situ* hybridizations of oyster tissue sections to visualize *H. costale* plasmodia and prespore stages; it did not hybridize with oyster (*C. virginica*) or other haplosporidans (*H. nelsoni, H. louisiana*, or *Minchinia teredinis*). DNA-based diagnostics for *H. costale*, in conjunction with molecular tools previously developed for *H. nelsoni*, have overcome limitations of histological examination. From *in situ* hybridizations using both probes, some Virginia oysters previously diagnosed with *H. costale* were found to have mixed infections consisting of approximately 80 to 90% *H. costale* plasmodia and 10 to 20% *H. nelsoni* plasmodia. Plasmodia of *H. costale* were not found in epithelial tissue, only in connective tissue. In addition, use of the DNA probe confirmed the presence of *H. costale* plasmodia in Virginia oysters collected in the fall, an unprecedented seasonality for an advanced *H. costale* infection.

KEY WORDS: *in situ* hybridization, small subunit ribosomal DNA, *Haplosporidium nelsoni, Haplosporidium costale*, eastern oyster, *Crassostrea virginica*, parasites

INTRODUCTION

Haplosporidium nelsoni Haskin, Stauber, and Mackin (MSX disease) and *Haplosporidium costale* Wood and Andrews (SSO disease) are morphologically similar pathogens of the eastern oyster, *Crassostrea virginica* Gmelin, that occur along the East Coast of the United States. *Haplosporidium costale* is generally thought to be restricted to high salinity bays (>25 ppt) along the open coast from Virginia to Maine; it is rare in the Delaware Bay and in the Chesapeake Bay (Andrews & Castagna 1978; Andrews 1988). *Haplosporidium nelsoni* occurs from Florida to Maine in both estuarine and oceanic habitats where the salinity is greater than about 10 ppt (Haskin & Andrews 1988). Thus, the distribution of the two pathogens overlaps in high salinity areas from Virginia to Maine.

If spores are present the parasites are easy to distinguish because *H. nelsoni* sporulates only in the epithelium of the digestive diverticula, whereas *H. costale* sporulates throughout the connective tissue of most organs (Couch 1967, Andrews & Castagna 1978). Moreover, spores of *H. nelsoni* are about twice the size of *H. costale* spores (Couch 1967). However, in the absence of spores, differentiation of the two parasites is very difficult, if not impossible. According to Couch (1967) plasmodia stages of both *H. nelsoni* and *H. costale* occur in epithelial and connective tissues in both mixed and single infections, so location of plasmodia is not helpful. *Haplosporidium costale* has a very restricted seasonality, with plasmodia present from March through June and spore stages present during May and June (Andrews et al. 1962, Andrews & Castagna 1978). However, plasmodia stages of *H. nelsoni* may also be common during the spring (Andrews 1982). Morphology of plasmodia has apparently been used to distinguish the species, with some difficulty. Couch and Rosenfield (1968) conducted a comparative study of *H. costale* and *H. nelsoni* in Chincoteague Bay, Virginia. They state that diagnoses of the two parasites in living oysters was based on recognition of the plasmodium, but they do not give any criteria used to distinguish the plasmodia of the two species. Mixed infections of *H. nelsoni* and *H. costale* were observed during the same study (Couch 1967), but they were based on the presence of spores of both species. However, criteria for distinguishing plasmodia of *H. nelsoni* and *H. costale* were provided (Couch 1967). They included: nuclear membranes of *H. costale* usually not as sharply defined or distinct as those of *H. nelsoni* and nucleoli (endosomes) of *H. costale* nuclei proportionately larger, less distinct, more diffuse, and more central than nucleoli of *H. nelsoni*. Andrews and Castagna (1978) stated that all stages of *H. costale* average smaller than those of *H. nelsoni*, but they went on to say that no definitive characters have been found with Harris hematoxylin and eosin (HHE) stain to distinguish *H. costale* and *H. nelsoni* plasmodia.

The specificity of molecular diagnostic tools, especially DNA probes used in *in situ* hybridizations, make them ideal for distinguishing morphologically similar species. Such tools are invaluable in elucidating certain ecological aspects of parasites that are difficult using traditional techniques (Burreson et al. 2000). Molecular diagnostic tools have been developed for *H. nelsoni* (Stokes & Burreson 1995, Stokes et al. 1995a). Specific polymerase chain reaction (PCR) primers have been developed for *H. costale* (Ko et al. 1995), but a DNA probe for that species has not been developed. Here we develop molecular diagnostic tools for *H. costale* and use the DNA probe in conjunction with an *H. nelsoni* DNA probe to identify mixed plasmodial infections of the two species. In addition, the molecular tools provided unexpected new information on the seasonality of *H. costale* in Virginia.

MATERIALS AND METHODS

DNA Sequences and Oligonucleotide Synthesis

The SSU rDNA sequences of *H. costale, H. nelsoni*, and *C. virginica* (GenBank accession AF387122, U19538, and X60315,

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respectively) were aligned using the MacVector software package (Oxford Molecular Group) and regions unique to *H. costale* were identified. PCR primers SSO-A (5′-CACGACTTTGGCAGTTAGTTTTG-3′) and SSO-B (5′-CGAACAAGCGCTAGCAGTACAT-3′) and DNA probe SSO1318 (same sequence as SSO-B, 5′ end labeled with digoxigenin) were commercially synthesized (Genosys Biotechnologies).

PCR Amplification

PCR reaction mixtures contained reaction buffer (10 mM Tris, pH 8.3; 50 mM KCl; 1.5 mM MgCl; 10 μg/mL gelatin), 400 μg/mL bovine serum albumin, 25 pmoles each of SSO-A and SSO-B, 200 μM each of dATP, dCTP, dGTP, dTTP, 0.6 units Ampli*Taq* DNA polymerase (Perkin–Elmer), and template DNA in a total volume of 25 μL. The reaction mixtures were cycled in a GeneAmp PCR System 9600 thermal cycler (Perkin–Elmer) 35 times at 94°C for 30 sec, 59°C for 30 sec, and 72°C for 1.5 min with a final extension at 72°C for 5 min. PCR reaction mixtures and cycling conditions for *H. nelsoni* were identical, except the primers were MSX-A′ and MSX-B (Renault et al. 2000, Stokes et al. 1995a). An aliquot (10% of reaction volume) of each PCR reaction was checked for amplification product(s) by agarose gel electrophoresis and ethidium bromide staining.

PCR Specificity and Sensitivity

Primer specificity was tested in PCR reactions using cloned SSU rDNA from *H. costale*, *H. nelsoni*, *Haplosporidium louisiana* Sprague, and *Minchinia teredinis* Hillman, Ford, and Haskin, and genomic DNA from *Haplosporidium lusitanicum* Azevedo, *Minchinia chitonis* (Lankester), *Minchinia tapetis* (Vilela), and uninfected *C. virginica*. Preparation of the cloned SSU rDNAs were described previously (Stokes et al. 1995a). Hatchery-reared juvenile *C. virginica* were collected in July 1999, and genomic DNA was tested for the presence of *H. nelsoni* by PCR, as described previously (Stokes et al. 1995a). Limpets, *Helcion pellucidus*, were collected from Cap de La Hague, near Cherbourg, France in September 1998 and screened for the presence of *H. lusitanicum* spores. Chitons, *Lepidochitona cinereus*, were collected from Wembury Bay, near Plymouth, England in September 1996 and screened for the presence of *M. chitonis* spores. *Minchinia tapetis*-infected clams, *Ruditapes decussatus* (L.), collected from Vilalonga in the Ria de Arousa, Galicia, Spain, in 1997 were kindly supplied by Antonio Villalba. Spores were concentrated from infected tissues and DNA extractions from spores and from *C. virginica* were performed with mechanical grinding followed by detergent lysis, as described previously (Stokes et al. 1995b). Primer sensitivity to homologous target DNA was determined with tenfold serial dilutions from 100 pg to 1 fg of cloned *H. costale* SSU rDNA.

Histology

Tissue samples were preserved in Davidson's AFA for at least 24 h. Fixed tissues were embedded in paraffin, sectioned 5–6-μm thick, and placed on positively charged slides (Fisher Scientific) for *in situ* hybridization or hematoxylin and eosin (H&E) staining. Tissue sections were kept in order as they were cut, and the consecutive sections were numbered on the slides. The microtome blade and forceps were cleaned with xylene between samples to prevent carry-over DNA contamination.

In Situ *Hybridization (ISH)*

Tissue sections for ISH were processed as described previously (Stokes & Burreson 1995), except hybridization solution contained 5 ng/μL SSO1318 DNA probe or 2 ng/μL MSX1347 DNA probe and the addition of Bismarck Brown Y counterstain after the nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) color development. Slides were washed with TE buffer (10mM Tris, pH 8.0; 1mM EDTA), then with dH_20 to stop the NBT/BCIP color development. Tissue sections were stained with 1% Bismarck Brown Y (Sigma Chemical) for 1 min, then rinsed three times with dH_20. The slides were coverslipped with GVA Mounting Solution (Zymed Laboratories) and examined by light microscopy. Negative control ISH consisted of dH_20 instead of DNA probe in the hybridization solution. Consecutive tissue sections of all samples were processed in the following order: section 1, stained with H&E; section 2, ISH with SSO1318; section 3, ISH with MSX1347, section 4, ISH with no probe.

DNA Probe Specificity

In situ hybridization with both DNA probes SSO1318 and MSX1347 were performed on four *C. virginica* that had been diagnosed by histological examination as infected only with *H. costale* (Table 1). The Virginia Marine Resources Commission (VIMS) Oyster Disease Archive reference numbers for these oysters, embedded in paraffin, are 177,822, 181,676, 181,677, and 196,774. All of these oysters were collected at Wachapreague, VA, on the sea side of Virginia's Eastern Shore, the type locality (Wood and Andrews 1962) for *H. costale*. To demonstrate the

TABLE 1.

Samples tested with in situ hybridizations using DNA probes SSO1318 and MSX1347.

Archive Sample Number	Collection Date	Diagnosis by Histological Examination	Diagnosis by *in situ* Hybridization
177,822	May 1988	*H. costale* heavy (spores present)	*H. costale* heavy; *H. nelsoni* heavy
181,676	May 1989	*H. costale* heavy (spores present)	*H. costale* heavy
181,677	May 1989	*H. costale* heavy (spores present)	*H. costale* heavy; *H. nelsoni* rare
196,774	October 1994	tentative *H. costale* heavy*	*H. costale* heavy; *H. nelsoni* light

* Diagnosis of 196,774 was uncertain. The infective agent appeared to be *H. costale*, however, such seasonality of an advanced infection was unprecedented.

Histological examination column indicates parasites identified in tissue sections and infection levels of original diagnoses. In situ hybridization column indicates parasites identified in tissue sections and infection levels with DNA probes. All samples were collected from the vicinity of Wachapreague, Virginia.

specificity of probe SSO1318, ISH was performed on sections of *C. virginica* tissues infected with *H. nelsoni* and *Perkinsus marinus* (Mackin, Owen, and Collier), of shipworm (*Teredo* sp.) tissue infected with *M. teredinis*, and of mud crab (*Panopeus* sp.) tissue infected with *H. louisiana*.

RESULTS

Specificity and Sensitivity of PCR Primers

The *H. costale* PCR primer pair SSO-A and SSO-B amplified a 557 bp region of the *H. costale* small subunit rDNA (Fig. 1A), targeting bases 784 to 1340 of that gene. The primers did not amplify DNA from oyster or from the haplosporidans *H. nelsoni*, *H. louisiana*, *H. lusitanicum*, *M. teredinis*, *M. chitonis*, or *M. tapetis* (Fig. 1A). The PCR product was readily detected after amplification of 100 fg to 100 pg of cloned *H. costale* SSU rDNA; 10 fg of template DNA was amplified, but the product band was very faint in the agarose gel (Fig. 1B).

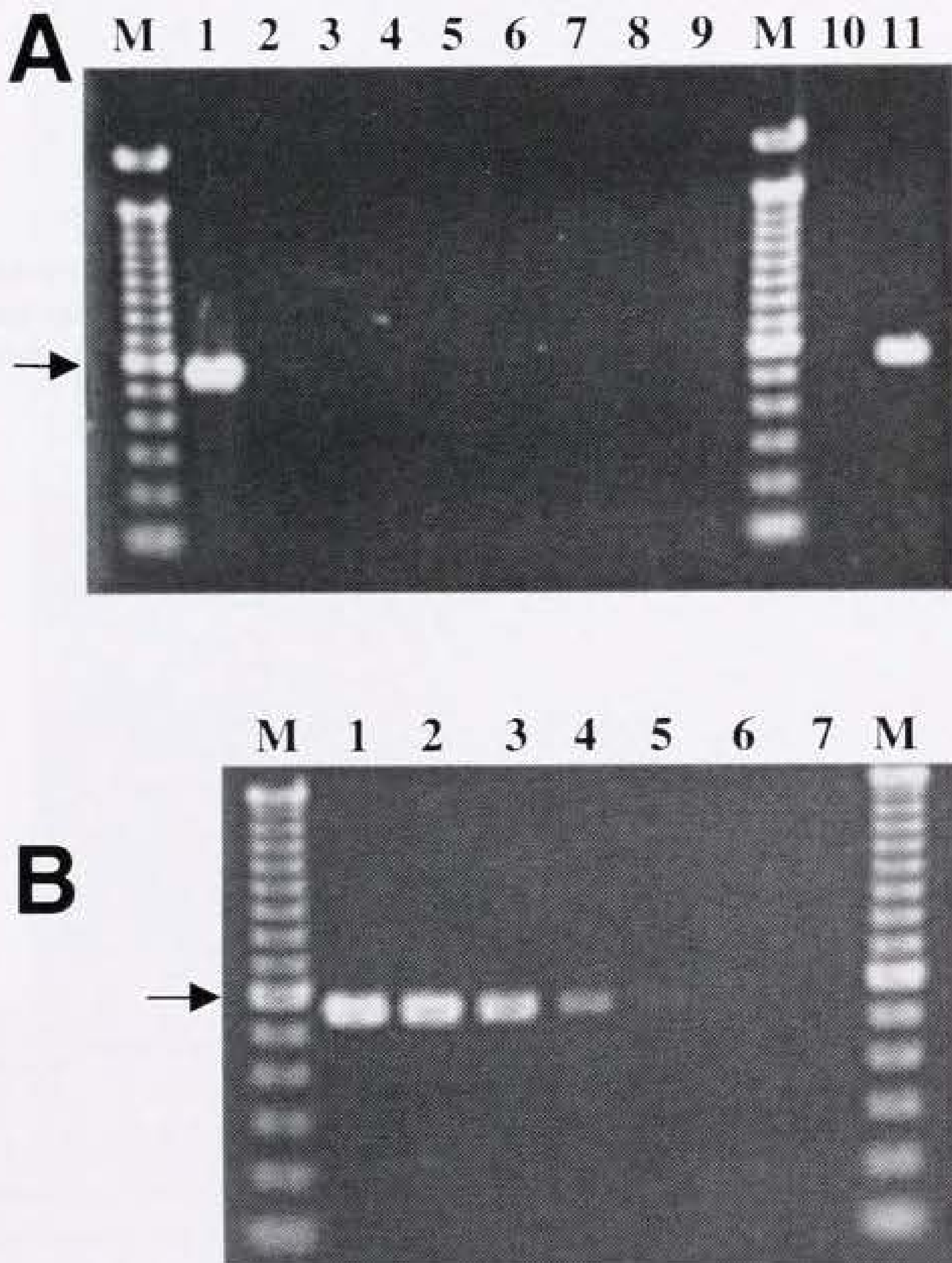

Figure 1. Specificity and sensitivity of *Haplosporidium costale* PCR primers. (A) Specificity. Lanes M, 100 bp ladder size marker, arrow indicates 600 bp; lane 1, cloned *H. costale* SSU rDNA; lane 2, cloned *H. nelsoni* SSU rDNA; lane 3, *H. lusitanicum* genomic DNA; lane 4, cloned *H. louisiana* SSU rDNA; lane 5, cloned *Minchinia teredinis* SSU rDNA; lane 6, *M. chitonis* genomic DNA; lane 7, *M. tapetis* genomic DNA; lane 8, uninfected *Crassostrea virginica* genomic DNA; lane 9, no DNA control; lane 10, *H. nelsoni*-infected *C. virginica* genomic DNA; lane 11, *H. costale*-infected *C. virginica* genomic DNA. (B) Sensitivity. PCR amplification products using *H. costale* primers SSO-A and SSO-B against serial dilutions of cloned *H. costale* SSU rDNA template. Lanes M: 100 bp ladder size marker, arrow indicates 600 bp; lane 1: 100 pg template DNA; lane 2: 10 pg; lane 3: 1 pg; lane 4: 100 fg; lane 5: 10 fg; lane 6: 1 fg; lane 7: no DNA control.

Specificity of DNA Probe

One of the candidate *H. costale* probes, designated SSO1318, was found to be sensitive and specific for *H. costale* in *in situ* hybridizations of tissue sections. Optimal hybridization required 5 ng/μL SSO1318 and incubation at 42°C. The SSO probe readily detected *H. costale* plasmodia and immature spores in sporocysts in ISH of oyster tissue with virtually no background, as indicated by the cells that stained dark purple to black (Fig. 2). DNA probe SSO1318 did not hybridize with oyster tissue (*C. virginica*), the oyster pathogen *P. marinus*, or the haplosporidans *H. nelsoni*, *H. louisiana*, and *Minchinia teredinis* (Fig. 3).

Differential Diagnosis using DNA Probes

Four oysters previously diagnosed by routine histological examination of H&E-stained paraffin sections as infected with *H. costale*, but not *H. nelsoni* (Table 1) were subjected to ISH using separate DNA probes for *H. costale* and *H. nelsoni*. Plasmodia and immature spores in tissues of all four oysters hybridized with the SSO probe (Figs. 4–6), thus supporting the histological diagnoses. However, some plasmodia in three of the four oysters did not hybridize with the *H. costale* probe, but instead hybridized with the *H. nelsoni* probe (Figs. 4–6). These mixed infections of *H. costale* and *H. nelsoni* were not distinguishable nor detectable by histological examination in part because only plasmodial stages of *H. nelsoni* were present, but they were readily apparent by *in situ* hybridization (ISH) using the species-specific DNA probes. Even a light infection of *H. nelsoni* plasmodia, scattered among a heavy infection of *H. costale* was easily detected using a DNA probe (Fig. 4E). In addition, the *H. costale* probe enabled discrimination of early and maturing plasmodia, the latter, which have vacuolated cytoplasm (Wood & Andrews 1962). The vacuoles within the stained plasmodia are easily seen at low power in ISH with the *H. costale* probe (Fig. 5B). *Haplosporidium nelsoni* plasmodia were found in both connective tissue and epithelia (Figs. 4E, F; 5C; 6C); *H. costale* plasmodia were located throughout the connective tissue but not in the epithelium of the four oysters examined (Figs. 4C, D; 6B).

One oyster collected in October 1994 seemed to be infected with *H. costale* based on the presence of plasmodia and appropriately sized immature spores within sporocysts scattered throughout the connective tissue as determined by stained paraffin sections (Table 1). However, the diagnosis was recorded as tentative, because advanced infections of *H. costale* were known only from April to June (Andrews & Castagna 1978) and none had ever been reported from the fall season. *In situ* hybridizations using both *H. costale* and *H. nelsoni* DNA probes confirmed a mixed infection of the two parasites (Fig. 6), thus documenting unprecedented timing of an advanced *H. costale* infection. Plasmodia and sporocysts of *H. costale* were abundant in connective tissue (Fig. 6B), but not in epithelium; plasmodia of *H. nelsoni* occurred in epithelium (Fig. 6C) but not in connective tissue.

DISCUSSION

The PCR primers SSO-A and SSO-B and the DNA probe SSO1318 were sensitive and specific for the target organism, *H. costale*. Another set of PCR primers for *H. costale* was previously reported by Ko et al. (1995); however, we chose to target a different region of the SSU rDNA. The two regions targeted by the probe and primers described here are highly variable within the phylum Haplosporidia, accessible for probe hybridization and have

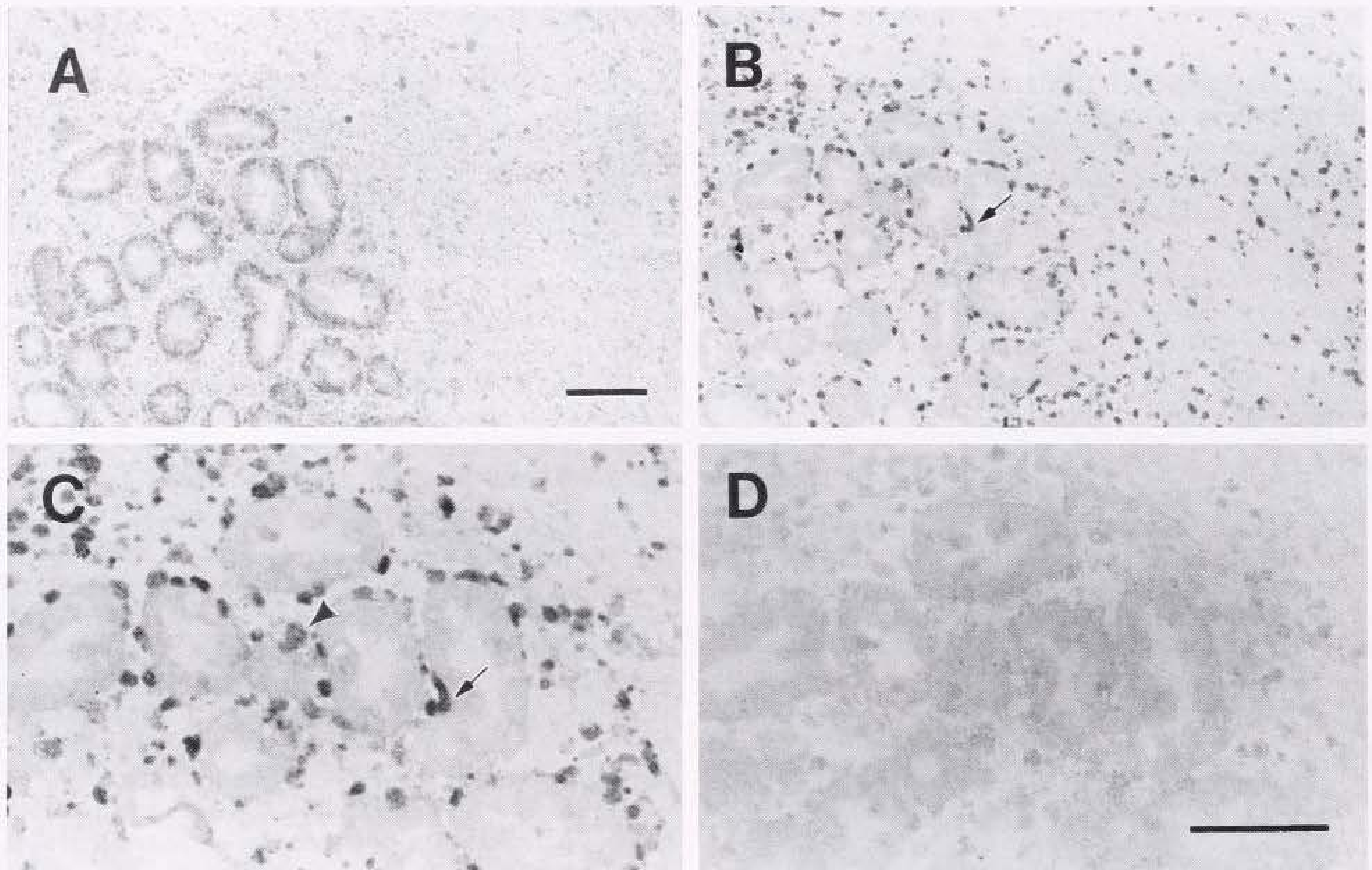

Figure 2. Consecutive histological sections of *H. costale*-infected *C. virginica* tissue (#181,676) showing plasmodia and sporocysts containing immature spores in the connective tissue. (A) Hematoxylin and eosin (H&E) stain. Bar = 100 µm and also applies to B. (B) *In situ* hybridization (ISH) with *H. costale* DNA probe. Arrow points to plasmodium enlarged in C. (C) ISH at higher magnification, arrow points to same plasmodium indicated in B. (D) ISH with *H. nelsoni* DNA probe. Bar = 100 µm and also applies to C.

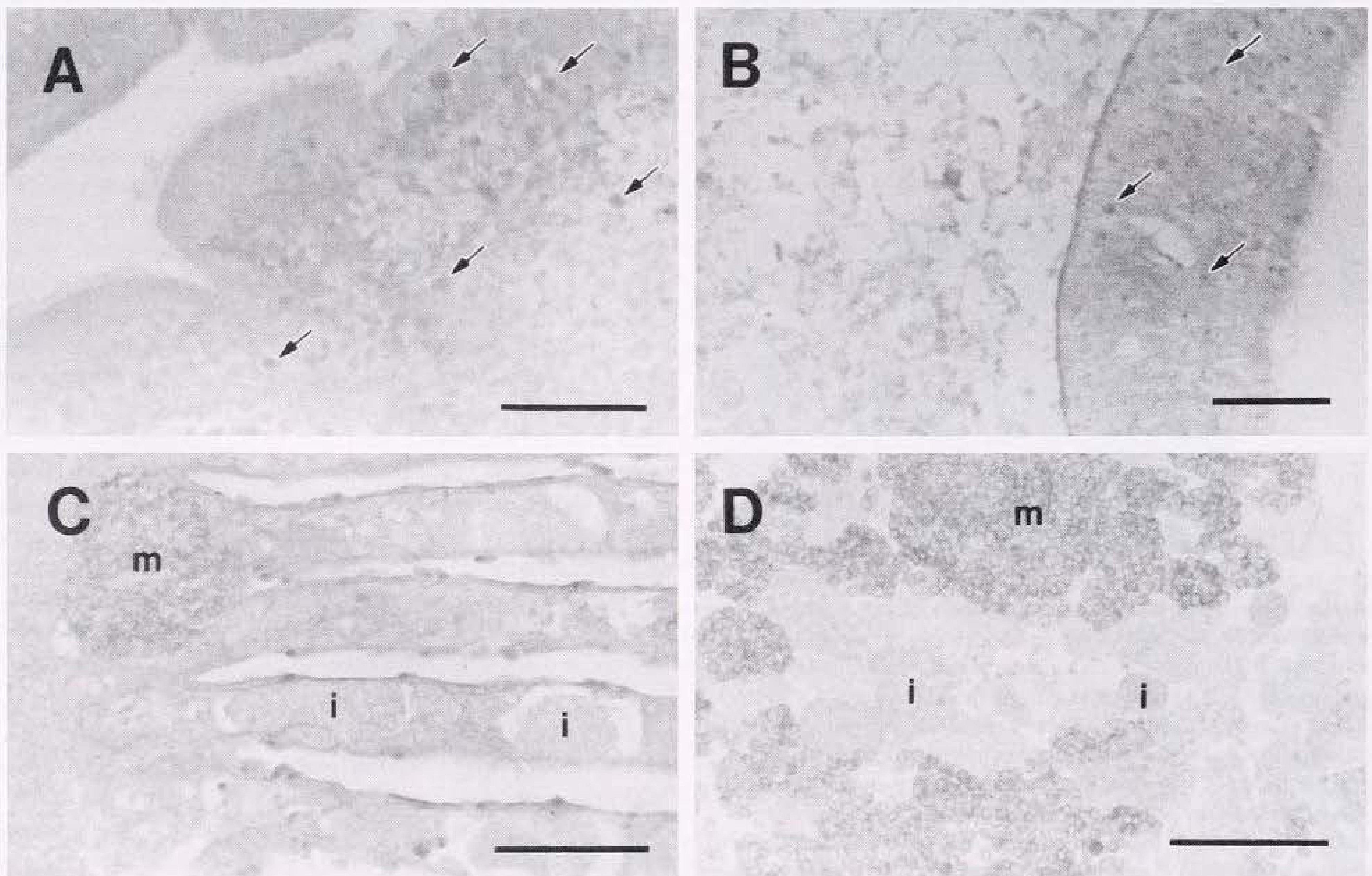

Figure 3. Lack of *in situ* hybridization (ISH) of various other parasites in histological sections demonstrating specificity of the *H. costale* DNA probe. (A) *H. nelsoni*-infected *C. virginica* tissue, arrows indicate some of the plasmodia present. Bar = 100µm. (B) *Perkinsus marinus*-infected *C. virginica* tissue, arrows indicate some of the cells present in the epithelium. Bar = 60 µm. (C) *M. teredinis*-infected *Teredo* sp. tissue, with immature (i) and mature (m) spores. Bar = 100 µm. (D) *H. louisiana*-infected *Panopeus* sp. tissue, with immature (i) and mature (m) spores. Bar = 100 µm.

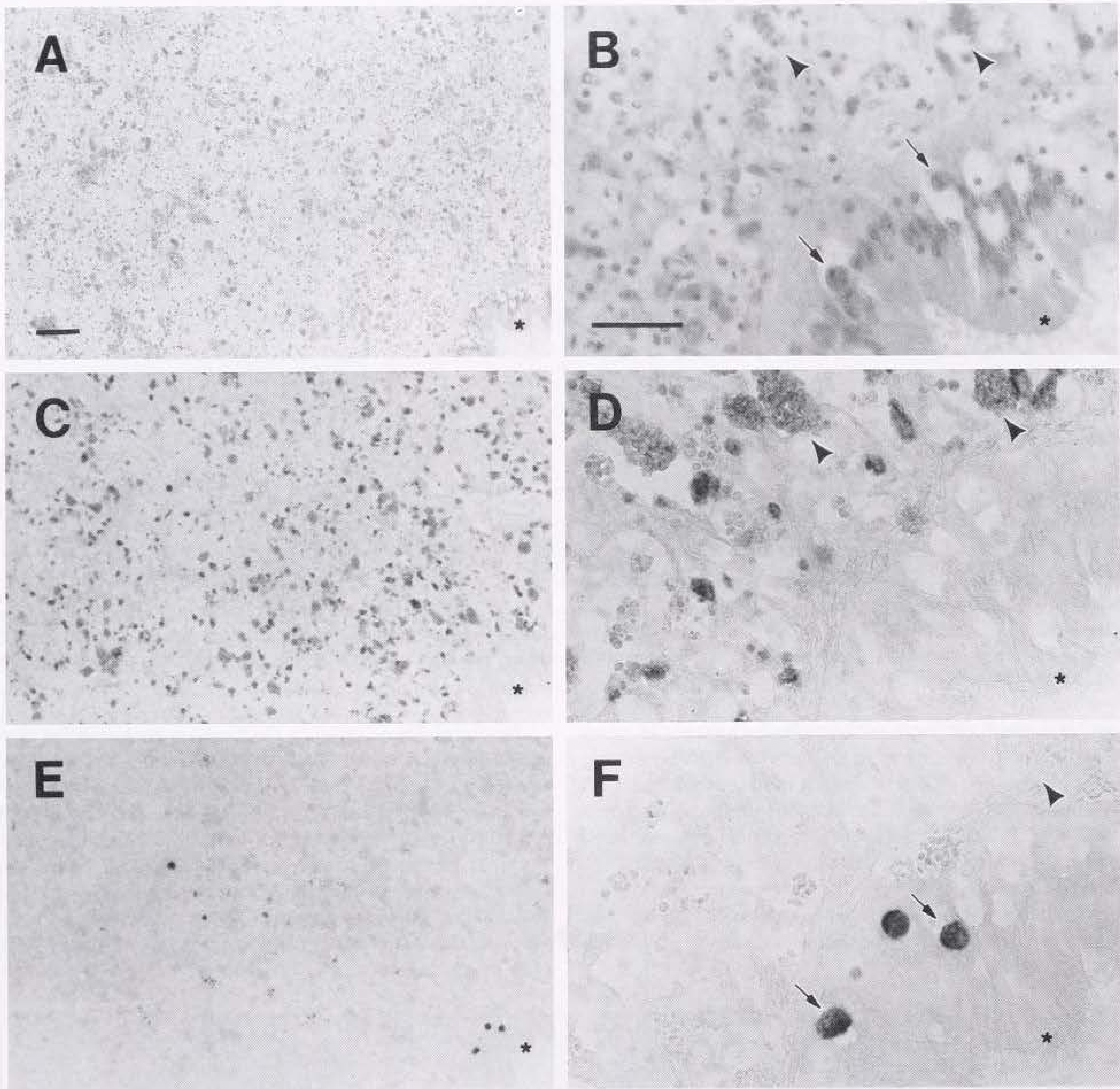

Figure 4. ***In situ*** **hybridization (ISH) of consecutive histological sections of oyster tissue (#181,677) collected in May 1989 from Virginia's Eastern Shore with a mixed haplosporidan infection. A, C, E all show the same area; B, D, F are higher magnifications of A, C, and E, respectively. Asterisk in lower right of each figure indicates the same epithelial lobe. (A) Hematoxylin and eosin (H&E) stain. Bar = 60 µm and also applies to C and E. (B) Hematoxylin and eosin (H&E) stain showing** ***H. nelsoni*** **plasmodia (arrows) in the epitheliium and some of the** ***H. costale*** **sporocysts (arrowheads) in the connective tissue. Bar = 30 µm and also applies to D and F. (C, D) ISH with** ***H. costale*** **DNA probe of same region in A and B showing positive reaction with** ***H. costale*****, but not** ***H. nelsoni*****. Arrowheads in D point to same** ***H. costale*** **sporocysts as in B. (E, F) ISH with** ***H. nelsoni*** **DNA probe of same region in A and B showing positive reaction with** ***H. nelsoni*** **plasmodia, but not with** ***H. costale*** **sporocysts. Note ability of DNA probe to identify rare** ***H. nelsoni*** **plasmodia in a heavy** ***H. costale*** **infection. Arrows in F indicate hybridization of** ***H. nelsoni*** **plasmodia in the epithelium as shown in B; arrowhead in F indicates lack of reaction with** ***H. costale*** **sporocyst shown in B and D.**

been used successfully for *H. nelsoni*-specific diagnostics (Stokes & Burreson 1995, Stokes et al. 1995a). The *H. costale* probe hybridized with *H. costale* plasmodia and immature spores, but not with mature spores, the same hybridization pattern as with the MSX probe with *H. nelsoni* (Stokes & Burreson 1995). In ISH of oyster samples from France, the SSO1318 probe did not hybridize with the Pacific oyster *C. gigas* nor with a haplosporidian infecting that host (Renault et al. 2000).

Mixed infections of *H. costale* and *H. nelsoni* that have not advanced to sporulation can now be diagnosed with confidence using these new tools. The plasmodia that hybridized with the *H. nelsoni* probe were not the same plasmodia that hybridized with the *H. costale* probe; although, these plasmodia were indistinguishable by traditional histological examination of stained tissue sections. The mixed parasite infections described here were originally diagnosed as being only *H. costale*. This diagnosis was undoubtedly made because of the preponderance of *H. costale* plasmodia and immature spores as compared to the relatively light infections of *H. nelsoni* and also because spores of *H. nelsoni* were absent. Couch (1967) reported finding mixed infections of *H. cos-*

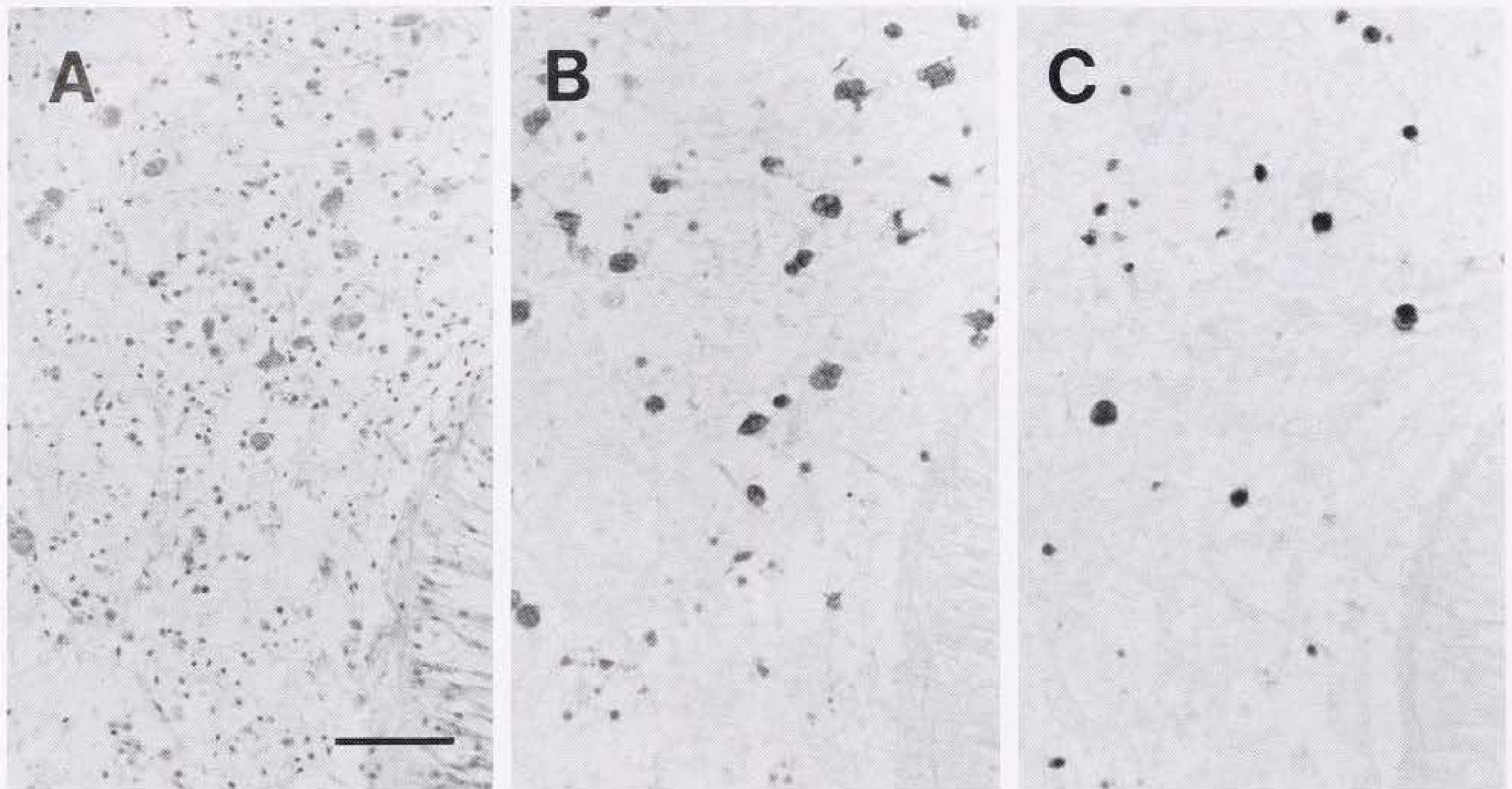

Figure 5. *In situ* **hybridization (ISH) of consecutive histological sections of oyster (#177,822) collected in May 1988 from Virginia's Eastern shore with mixed haplosporidan infection illustrating ease of plasmodia differentiation with DNA probes. (A) Hematoxylin and eosin (H&E) stain. Bar = 50 µm and applies to B and C. (B) ISH with** *H. costale* **DNA probe of same area shown in A. (C) ISH with** *H. nelsoni* **DNA probe of same area shown in A and B.**

tale and *H. nelsoni*, but they were based on the presence of spores of both species. In oysters tested to date with DNA probes, we have not observed *H. costale* plasmodia in the epithelium.

The inability to distinguish nonsporulating mixed haplosporidian infections by traditional histological examination may have skewed epizootiology data for high salinity regions in the past. It is possible that *H. nelsoni* has been more common in Virginia oysters in high salinity than previously reported. If so, this may raise questions about past disease data and oyster mortality attributed to *H. costale*.

Results of diagnoses using DNA probes have revealed an unprecedented seasonality of *H. costale* infections. The original diagnosis of *H. costale* infection in oyster #196,774 in October 1994 was uncertain. The plasmodia and immature spores throughout the connective tissue looked like *H. costale*, but the timing of this advanced infection was unusual. Epizootiological studies of *H. costale* had established the annual infection cycle as quite predictable. Clinical infections appear in the spring, as early as March, with sporulation and oyster mortality primarily in May and June. New infections occur before August 1st but remain subclinical

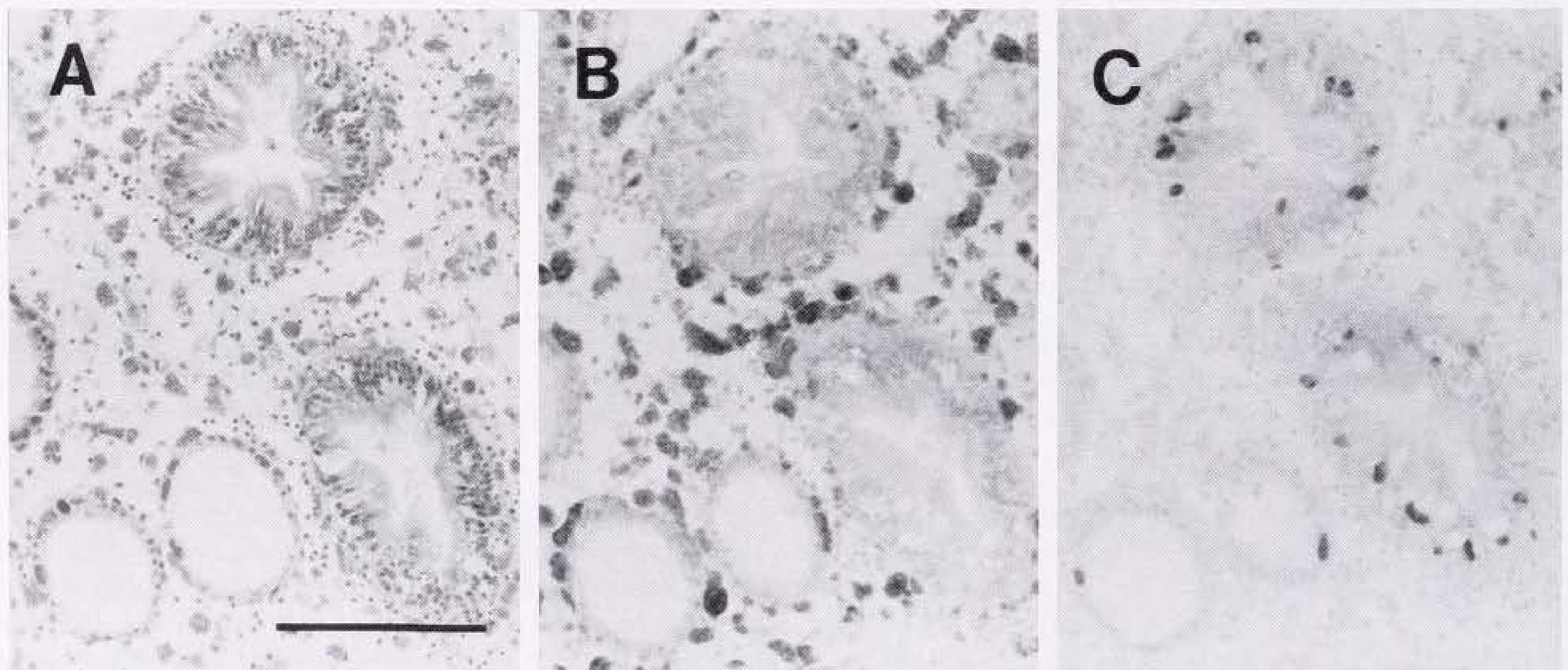

Figure 6. *In situ* **hybridization (ISH) of consecutive histological sections of oyster (#196,744) collected in October 1994 from Virginia's Eastern Shore with mixed haplosporidan infection. (A) Hematoxylin and eosin (H&E) stain. Bar = 100 µm and applies to B and C. (B) ISH with** *H. costale* **DNA probe of same area shown in A. Note** *H. costale* **plasmodia in connective tissue, but not in epithelium. (C) ISH with** *H. nelsoni* **DNA probe of same area shown in A and B. Note** *H. nelsoni* **plasmodia in epithelium, but not in connective tissue.**

until the following spring (Couch & Rosenfield 1968, Andrews & Castagna 1978, Andrews 1988). Andrews and Castagna (1978) reported that numerous samples of seaside Virginia oysters from summer through winter revealed no *H. costale* infections. Diagnosis by DNA probes of oyster 196,774 confirmed the *H. costale* diagnosis, as about 80% of the plasmodia hybridized with the SSO1318 probe, but also revealed it as a mixed infection, because about 20% of the plasmodia hybridized with the MSX1347 probe. This *H. costale* infection, where the parasite's identity was confirmed by DNA-based diagnostics, did not meet historical criteria for SSO disease suggesting that the seasonality and epizootiology of this pathogen must be re-examined.

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