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NOTE

Conservation of sequence in the internal transcribed spacers and 5.8S ribosomal RNA among geographically separated isolates of parasitic scuticociliates (Ciliophora, Orchitophryidae)*

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ABSTRACT: Nucleotide sequence from the internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene from the ribosomal RNA gene cluster of isolates of the scuticociliate *Orchitophrya stellarum* from 4 asteroid hosts were compared. Surprisingly, these data (495 bp) were identical for *O. stellarum* isolated from the testes of *Asterias amurensis* from Japan; *Pisaster ochraceus* from British Columbia, Canada; *Asterias rubens* from The Netherlands; and *Asterias vulgaris* from Prince Edward Island, Canada. These sequence data were compared to those from 3 scuticociliates which parasitise crustaceans: *Mesanophrys pugettensis*, *M. chesapeakensis* and *Anophryoides haemophila*. No difference was found in this region between the nucleotide sequence of *M. pugettensis* and *M. chesapeakensis*. The sequence of *Mesanophrys* spp. differed by 9.2% in the ITS1 and 4.7% in the ITS2 from that of *O. stellarum*. The sequence from the ITS1 (135 bp) and ITS2 (233 bp) of *A. haemophila* differed by 42.6 and 20.5% respectively from those of *O. stellarum*. Therefore, nucleotide sequence of the ITS regions in these scuticociliates is highly conserved.

KEY WORDS: *Orchitophrya* · *Mesanophrys* · *Anophryoides* · ITS · Scuticociliate

The taxonomy of scuticociliates is based primarily on oral structures and somatic kinetids. However, oral structures are plastic even within a class and are considered 'frail characters' to separate even subphyla (Greenwood et al. 1991). Somatic kinetids are arguably the most conservative of all ciliate structures and can provide insight into phylogenetic relationships (Lynn & Small 1988) but are limited in their ability to discriminate close relatives. Molecular data can be used to supplement classical morphological taxonomy. In particular, the internal transcribed spacers (ITS1 and ITS2) in the ribosomal RNA (rRNA) gene cluster have been used to discriminate the alveolates which comprise the apicomplexans (Cai et al. 1992, Goggin 1994, Schlotterer et al. 1994, Homan et al. 1997), dinoflagellates (Hudson & Adlard 1996) and ciliates (Diggles & Adlard 1997). We investigated the utility of this region to discriminate orchitophryid scuticociliates which are geographically separated.

In particular, we chose the parasitic scuticociliate *Orchitophrya stellarum* which was first described within the testes of the asteroid seastar *Asterias rubens* from France (Cépède 1907). The same species has since been recorded from the Pacific and Atlantic Oceans: from *A. forbesi* from Connecticut, USA (Burrows 1936), *A. vulgaris* from Prince Edward Island and Quebec, Canada (Smith 1936, Claereboudt & Bouland 1994), *Sclerasterias richardii* from the Mediterranean Sea (Febvre et al. 1981), *Pisaster ochraceus* from British Columbia, Canada (Leighton et al. 1991), and *A. amurensis* from Japan (Byrne et al. 1997, Goggin & Bouland 1997). Few of these isolates have been adequately described and, to our knowledge, none have been deposited in museums. Therefore, the specific identity of these ciliates remains uncertain.

To determine the variation in the ITS regions between orchitophryid scuticociliates, we compared 4 wild isolates of *Orchitophrya stellarum* from around the globe with 3 scuticociliates which parasitise crustaceans and were held in laboratory cultures.

Materials and methods. Testes infected with *Orchitophrya stellarum* were collected from 4 asteroid seastars from the Pacific and Atlantic oceans: *Asterias rubens* in April 1997 from Wemeldinge, Oosterschelde, The Netherlands (n = 4); *A. vulgaris* in May 1997 from Murray River, Prince Edward Island, Canada (n = 3);

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Pisaster ochraceus in May 1997 from Seymour Bay, Bowen Island, British Columbia, Canada (n = 3); and *A. amurensis* in June 1996 from Usujiri, Hokkaido, Japan (n = 4). The infected testes were preserved in 100% ethanol.

Three scuticociliates kept in culture media (modified ATCC 1651 MA medium with the addition of 10% foetal bovine serum) since September 1995 were also collected: *Anophryoides haemophila* isolated from the lobster *Homarus americanus*; *Mesanophrys chesapeakensis* from the blue crab *Callinectes sapidus*; and *M. pugettensis* from the dungeness crab *Cancer magister*. These ciliates were kindly supplied by Dr R. J. Cawthorn, Atlantic Veterinary College, The University of Prince Edward Island, Canada. Cultures were centrifuged to concentrate the ciliates and the pellet was preserved in 100% ethanol.

Ethanol was removed and ciliates or tissue crushed in extraction buffer (50 mM Tris, pH 8.0, 0.7 M NaCl, 10 mM EDTA, 1% hexadecyltrimethylammonium bromide, 0.1% 2-mercaptoethanol) prior to incubation at 65°C for 1 h with 100 µg ml^{-1} proteinase-k added. DNA was extracted using phenol/chloroform, precipitated in ethanol and resuspended in double distilled water. A region of rDNA was amplified from all isolates by polymerase chain reaction (PCR) in 50 µl volumes using 20 ng DNA, 800 µm dNTPs (Promega, Wisconsin, USA) and 20 pm each primer (Bresatec, Adelaide, Australia). A forward primer (SB2) located in the 3' region of the small subunit (SSU) rRNA gene and a reverse primer (ITS2.2) located at the 5' region of the large subunit (LSU) gene were used in PCR (Goggin & Newman 1996). Other PCR reagents were as supplied in a Perkin-Elmer kit (California, USA) and used in the following concentrations: 1.25 Units AmpliTaq Gold, 0.1 mM $MgCl_2$ in buffer (5 mM Tris-HCl, 50 mM KCl, pH = 8.3). PCR were run for 30 cycles of: 94°C for 60 s, 56°C for 30 s and 72°C for 90 s with a final cycle of 94°C for 60 s, 55°C for 30 s and 72°C for 7 min. The amplified region comprised the 3' region of the SSU gene, the ITS1, 5.8S gene, ITS2 and 5' region of the LSU gene and was approximately 750 bp in all ciliates. DNA was purified using QiaQuick spin columns (QIAGEN Inc., Chatsworth) following the protocol recommended by the manufacturer. PCR products were sequenced using the same primers in a dye terminator sequencing reaction (Perkin-Elmer) and run on an acrylamide gel on an automated sequencer (ABI Prism 377 DNA sequencer). Sequences were collected from *Orchitophrya stellarum* isolated from 3 hosts at each site, 2 separate cultures of both *Mesanophrys chesapeakensis* and *Anophryoides haemophila*, and a single culture of *M. pugettensis*. Forward and reverse sequences were collected from all ciliate isolates.

Results and discussion. Surprisingly, there were no differences between nucleotide sequence from the ITS1 (140 bp), 5.8S (119 bp) and ITS2 (236 bp) rRNA of *Orchitophrya stellarum* isolated from *Asterias amurensis*, *A. rubens*, *A. vulgaris* and *Pisaster ochraceus* (Fig 1). The lack of variation was unexpected given the geographic and host spread of these isolates. Even more surprisingly, there were no differences between the ITS1 (139 bp) or the ITS2 (233 bp) of *Mesanophrys pugettensis* isolated from *Cancer magister* from the Pacific Ocean and *M. chesapeakensis* isolated from *Callinectes sapidus* from the Atlantic Ocean (Fig. 1). In contrast, the ITS sequence of the alga *Cladophora albida* from the Pacific Ocean differed by 21% from isolates in the Atlantic Ocean, while the sequence of isolates collected within either ocean were nearly identical (Bakker et al. 1992). Differences in the ITS regions have also been found between strains and species of ciliates. For example, the nucleotide sequence of 4 wild isolates of *Cryptocaryon irritans* differed by 0.6 to 4.1% in the ITS1 (Diggles & Adlard 1997) while that of 2 species of *Tetrahymena* differed by 3% in ITS1 and 10% in ITS2 (Engberg et al. 1990).

Furthermore, sequences from the ITS1 region of the ciliate *Cryptocaryon irritans* changed rapidly with passage in the laboratory and differed by 5.9% from the original isolate after approximately 1 yr (Diggles & Adlard 1997). In our study, no difference was found between the sequence of the ITS regions of *Mesanophrys pugettensis* and *M. chesapeakensis* which were held in culture for about 18 mo (Fig. 1).

In some cases, little variation is found in ITS sequence between strains and species of protistans. For example, isolates of the protistan *Perkinsus* from Australia and Portugal had identical ITS1 sequences and 0.8% difference in ITS2 sequences (Goggin 1994). There were also no differences in the ITS1 or ITS2 of 12 isolates of *Tritrichomonas foetus* and *T. suis* (Felleisen 1997). Therefore, the ITS regions may be conserved in some species and strains. Indeed, it appears that the ITS regions of the scuticociliates sequenced here are conserved despite considerable geographic and reproductive isolation.

The nucleotide sequence from the ITS regions of *Mesanophrys* spp. are more similar to the consensus sequence of *Orchitophrya stellarum* than is that of *Anophryoides haemophila*. These sequences were aligned by eye to give a consensus sequence of 141 bp for the ITS1, 119 bp for the 5.8S and 239 bp for the ITS2 (Fig. 1). The consensus sequence of *Mesanophrys* spp. differed by 9.2% in the ITS1 (8 transversions, 2 transitions, 1 addition, 2 deletions) and 4.7% in the ITS2 (1 transversion, 7 transitions, 3 deletions) from that of *O. stellarum* (Table 1). The ITS1 of *A. haemophila* differed by 42.6% (45 transversions, 8 transitions, 1 addition, 6 deletions) and the ITS2 (233 bp) by 20.5% (21 transversions, 19 transitions, 3 additions, 6 dele-

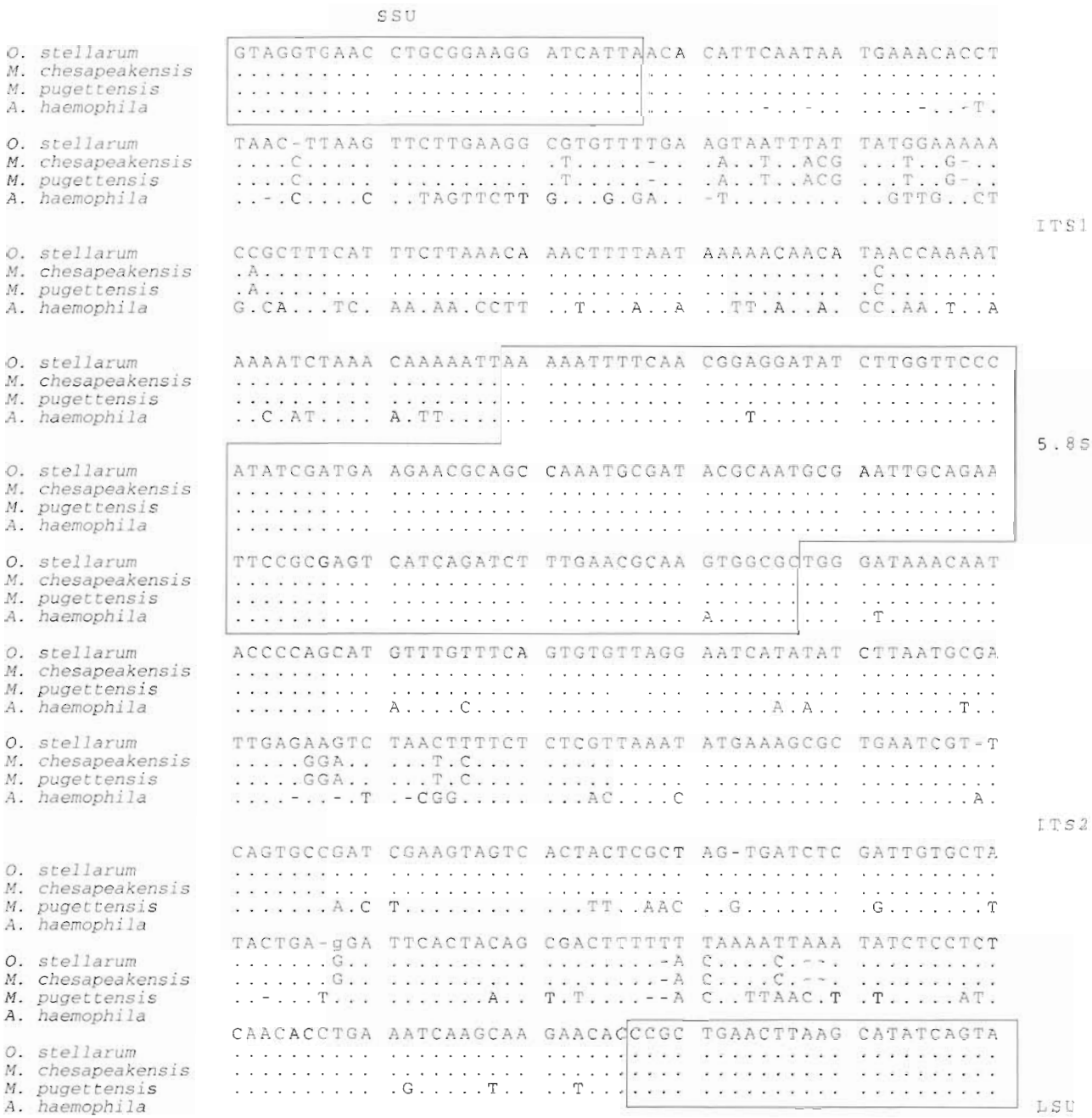

Fig. 1. Consensus nucleotide sequence data from the partial small subunit (SSU), internal transcribed spacer 1 (ITS1), 5.8S and ITS2 rRNA of the ciliate *Orchitophrya stellarum*, aligned with sequence from *Mesanophrys chesapeakensis* (AF107778), *M. pugettensis* (AF107777) and *Anophryoides haemophila* (AF107779). The data from *O. stellarum* are a consensus sequence of 4 geographically separate isolates from 4 seastar hosts: *Asterias amurensis* (from Japan, GenBank accession number AF107773), *A. rubens* (Netherlands, AF107776), *A. vulgaris* (Prince Edward Island, Canada, AF107774) and *Pisaster ochraceus* (British Columbia, Canada, AF107775). LSU: large subunit

tions) from that of *O. stellarum* (Table 1). Hudson & Adlard (1996) found a 33.5 to 77.3% difference in partial ITS1 sequence between isolates of the dinoflagellate *Hematodinium*. Thus, this region varies much less between the scuticociliates sequenced here than some other alveolates.

The GC (guanine + cytosine) content of the ITS1 does not equal that of the ITS2 in *Orchitophrya stellarum* (ITS1 = 24.3%; ITS2 = 38.1%), the consensus sequence from *Mesanophrys* spp. (ITS1 = 25.2%, ITS2 = 39.4%) or *Anophryoides haemophila* (ITS1 = 21.4%, ITS2 = 36.4%) which is unlike 19 other species of

Table 1. Nucleotide sequence differences of the internal transcribed spacer 1 (ITS1), 5.8S and internal transcribed spacer 2 (ITS2) rRNA gene between the consensus sequence of 4 isolates of *Orchitophrya stellarum* (*Os*), the consensus sequence from *Mesanophrys pugettensis* and *M. chesapeakensis* (*Mes*) and *Anophryoides haemophila* (*Ah*)

Species	Position differences	ITS1			5.8S			ITS 2		
		Os	*Mes*	*Ah*	*Os*	*Mes*	*Ah*	*Os*	*Mes*	*Ah*
Orchitophrya stellarum	%	0	9.2	42.6	0	0	1.7	0	4.7	20.5
	No. diff/total no.	0/140	13/141	60/135	0/119	0/119	2/119	0/236	11/239	49/233
Mesanophrys spp.	%		0	46.1		0	1.7		0	21.0
	No. diff/total no.		0/139	65/141		0/119	2/119		0/233	50/238
Anophryoides haemophila	%			0			0			0
	No. diff/total no.			0/135			0/119			0/233

fungi, plants and animals (Torres et al. 1990). However, there is also an imbalance in the GC content of the ITS1 and ITS2 in the ciliate *Tetrahymena thermophila* (ITS1 = 29.8% GC content, ITS2 = 43.3%) (Engberg et al. 1990), the dinoflagellate *Dictyostelium discoideum* (ITS1 = 28%, ITS2 = 43%) (Ozaki et al. 1984), the kelp *Alaria marginata* (ITS1 = 52%, ITS2 = 67%) and many plants (Saunders & Druehl 1993).

As expected, the 5.8S rRNA gene was highly conserved between these scuticociliates. The 5.8S sequence of *Mesanophrys* spp. was identical to the consensus sequence from the 4 isolates of *Orchitophrya stellarum* (119 bp, 45.4% GC content); there was 1 transition and 1 transversion between these sequences and that of *Anophryoides haemophila* (Table 1).

In conclusion, data from ITS are highly conserved and unlikely to discriminate species or strains of orchitophryid scuticociliates but may be useful to discriminate genera in this group.

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